REMARKS

Further and a favorable reconsideration is respectfully requested in view of the foregoing amendments and following remarks.

Thus, claim 1 has been amended to recite that the solution of 1,5-D-anhydrofructose is an aqueous solution of this material, based on the disclosure at page 6, lines 1-3 of the specification. The term "aqueous solution" is also used in the working examples.

The patentability of the presently claimed invention over the disclosure of the reference relied upon by the Examiner in rejecting the claims will be apparent upon consideration of the following remarks.

Thus, the rejection of claims 1-7 under 35 U.S.C. § 103(a) as being unpatentable over Elsser et al. (WO '060) is respectfully traversed.

This reference, at page 9, line 31 to page 10, line 4, discloses the following methods of manufacturing Ascopyrone P:

> "(1) Ascopyrone P may be produced by treating 1,5-anhydro-D-fructose with **non-aqueous** acid at elevated temperature, for example at 70°C.
> (2) Ascopyrones (for example, Ascopyrone P, T and M) may be produced from 1,5-anhydro-D-fructose by alkali treatment according to Ahmad, T., 1995." (Emphasis Added.)

The method (1) above makes use of "non-aqueous acid." Although examples of the non-aqueous acid are not given, it is obvious from the word "non-aqueous" that this acid **does not contain water**. Therefore, it can be said that the method (1) makes use of a non-aqueous solution of 1,5-anhydro-D-fructose in non-aqueous acid (if a solution is formed though it is unknown whether it is actually a solution or not).

The method in claim 1 of the present application and the method (1) of Elsser et al. differ from each other in the following points:

(i) As the examiner says, the pH of the solution is not specified in the method (1).

(ii) A reaction is carried out in a non-aqueous solution in the method (1) whereas a reaction is carried out in an aqueous solution in the method in claim 1 of the present application. That is, they basically differ from each other in reaction system.

Since it is known that an aqueous reaction completely differs from a non-aqueous

reaction, a person of ordinary skill in the art could not specify, with reasonable certainty, what the reaction temperature and pH should be in the aqueous reaction in claim 1 of the present application based on the method (1), more particularly, for manufacturing APT advantageously at a high yield as shown in Figs. 2 to 6 of the present application. In other words, since a person of ordinary skill in the art, having knowledge of the method (1) of the non-aqueous reaction system of Elsser et al., would understand that the method (1) cannot be adapted to an aqueous reaction, the art-skilled could not reasonably predict the aqueous reaction in claim 1 of the present application by relying on the method (1).

Further, as for the method (2) of Elsser et al., it is detailed in the thesis paper of Ahmad, T., 1995 (STUDIES ON THE DEGRADATION OF SOME PENTOSES AND OF 1,5-ANHYDRO-D-FRUCTOSE, THE PRODUCT OF THE STARCH-DEGRADATING ENZYME a-1,4-GLUCAN LYASE) cited in Elsser et al., and Paper [V] on page 6 of the thesis (Reactions of 1 , 5 -anhydro-D-fructose in alkaline aqueous solution). Applicants enclose herewith a copy of each of these documents (along with copies of Papers [I]-[IV] also referred to on page 6 of the theses) and will explain the method (2) hereinbelow.

The thesis of Ahmad, T., 1995 teaches in section 4.2 at page 27 that 1,5-anhydro-D-fructose (1,5-AF) is degraded in an aqueous alkali with reference to Paper [V] . Compound 1 in scheme 9 at page 27 of the document is 1,5-AF and compound 50 is Ascopyrone P (APP). Paper [V] teaches at page 2, line 4 from the bottom to page 3, line 2 that when products obtained by treating AF with aqueous NaOH (0.5M, 0.7 ml) at 25°C for 5 minutes and neutralizing it with HC1 are separated into two fractions by HPLC, compound 4 (APP, see page 5, scheme 1) is contained in the first fraction.

Since 1.5-AF is treated under a strong alkaline condition (0.5M, probably pH is almost 14) in the method (2) of Elsser et al., the formation of APP is observed by HPLC but the quantity of APP is very small (see Fig. 3 of the present application). Since the method (2) merely discloses a reaction at 25°C as described above, it is impossible to reasonably predict from method (2) that APP can be manufactured at a temperature higher than 100°C, especially at a high yield.

For these reasons, Applicants take the position that the presently claimed invention is clearly patentable over the Elsser et al. reference.

In addition, with regard to claims 6 and 7, the method of these claims clearly differs from Elsser et al. for the additional reason that, in the present invention, an antioxidant is used in the reaction for producing APP; whereas in Elsser et al., the antioxidant is added to the APP after the APP has been manufactured.

With particular regard to claim 7, the Examiner states that it is obvious to use an acid such as or including Applicants' claimed antioxidant, ascorbic acid, since Elsser et al. disclose that acid can be used.

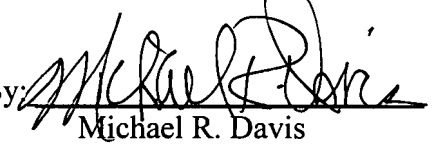
However, the mere fact that ascorbic acid (a species) is an acid (a genus) does not establish a presumption that the use of ascorbic acid is obvious. As specifically pointed out in MPEP 2144.08, Section II, the fact that a claimed species (or subgenus) is encompassed by a prior art genus is not sufficient by itself to establish a *prima facie* case of obviousness.

For these additional reasons, the subject matter of claims 6 and 7 is considered to be patentable over Elsser et al.

Therefore, in view of the foregoing amendments and remarks, it is submitted that the ground of rejection set forth by the Examiner has been overcome, and that the application is in condition for allowance. Such allowance is solicited.

Respectfully submitted,

Kazuhiro YOSHINAGA et al.

By: 

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October 22, 2007

STUDIES ON THE DEGRADATION OF SOME PENTOSES AND OF 1,5-ANHYDRO-D-FRUCTOSE, THE PRODUCT OF THE STARCH-DEGRADING ENZYME α-1,4-GLUCAN LYASE

by

Tania Ahmad

Doctoral dissertation to be publicly examined in Lecture Hall 1 of the Department of Chemistry, Swedish University of Agricultural Sciences, Arrheniusplan 8, Uppsala, on April 26, 1995 at 10.15 a. m., for the degree of Doctor of Philosophy. The discussion will be held in English.

Abstract: The formation of 2-furaldehyde and formic acid from pentoses in slightly acidic D_2O was studied by ^1H NMR spectroscopy. Increasing pD retarded the formation of 2-furaldehyde and increased its deuterium content at H-α and H-3. 2-Furaldehyde formed mainly via acyclic intermediates, with reversible formation of a 3-deoxypentosulose. The formic acid was obtained exclusively from the terminal pentose carbons, C-1 being somewhat more important than C-5.

While the formation of 2,3-dihydroxyacetophenone from aldopentoses or hexuronic acids under similar conditions was investigated, the structure of its previously isolated alicyclic precursor was revised to 3-acetyl-2,3,4-trihydroxycyclohexanone with *cis*-related hydroxyl groups.

Hydrolysis of (±)-*cis*- or (±)-*trans*-tetrahydro-2,5-dimethoxy-2-furaldehyde dimethyl acetal proceeded via 5,5-dimethoxy-4-oxopentanal to give (±)-*trans*-4,5-dihydroxy-2-cyclopentenone and its 5-*O*-methyl ether. The former product did not form reductic acid (2,3-dihydroxy-2-cyclopentenone) on treatment with dilute acid or base, an observation that is inconsistent with the tautomerization mechanism suggested in previous work.

The α-1,4-glucan lyase (EC 4.2.2.-), isolated from the red alga *Gracilariopsis lemaneiformis*, degraded starch/glycogen, forming 1,5-anhydro-D-fructose (1) from the non-reducing end groups. The substrate specificity, mode of action, and cleavage mechanism of the enzyme were studied by using amylose and its 1-^2H-, 2-^2H-, and 1-^{13}C-substituted analogues as substrates.

1',4-Anhydro-3-deoxy-2-*C*-(hydroxymethyl)-D-*threo*-pentonic acid was obtained from the degradation of **1** in aqueous alkali at room temperature. It was formed via the 4-deoxy-2,3-diulose. In alkali, **1** was oxidized by 3,5-dinitrosalicylic acid to 1',4-anhydro-2-*C*-(hydroxymethyl)-D-arabonic acid.

Keywords: 2-Furaldehyde, 2,3-dihydroxyacetophenone, reductic acid, 1,5-anhydro-D-fructose, 1',4-anhydro-3-deoxy-2-*C*-(hydroxymethyl)-D-*threo*-pentonic acid

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Department of Chemistry

Dissertation

Uppsala 1995

Doctoral Thesis

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1995

To my Parents

To Rawaz and Jamil

Contents

Abstract

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List of Papers

The following papers are included in this thesis, and they will be referred to by their Roman numerals:

[I] The formation of 2-furaldehyde and formic acid from pentoses in slightly acidic deuterium oxide studied by ^{1}H NMR spectroscopy. Tania Ahmad, Lennart Kenne, Kjell Olsson, and Olof Theander. *Carbohydr. Res.*, in press.

[II] On the formation of 2,3-dihydroxyacetophenone from pentoses or hexuronic acids. Tania Ahmad, Rolf Andersson, Kjell Olsson, and Olof Theander. *Carbohydr. Res.*, 247 (1993) 211-215.

[III] On the formation of reductic acid from pentoses or hexuronic acids. Tania Ahmad, Rolf Andersson, Kjell Olsson, and Eric Westerlund. *Carbohydr. Res.*, 247 (1993) 217-222.

[IV] α-1,4-Glucan lyase, a new class of starch/glycogen degrading enzyme. III. Substrate specificity, mode of action, and cleavage mechanism. Shukun Yu, Tania Ahmad, Lennart Kenne, and Marianne Pedersén. *Biochim. Biophys. Acta*, in press.

[V] Reactions of 1,5-anhydro-D-fructose in alkaline, aqueous solution. Tania Ahmad, Lennart Kenne and Marianne Pedersén. Manuscript.

1. INTRODUCTION

1.1. Nonenzymatic Degradation of Sugars

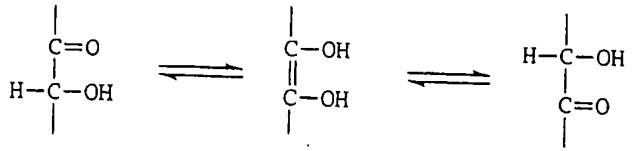
In the absence of enzymes, carbohydrates may be degraded in the presence of acids, bases or amines, such as proteins and amino acids. The degradation may also be accomplished simply by strong heating, as in the so-called caramelization of sugars [1]. Carbohydrate degradation is important in pulping [2], where it may result in discoloration and poor quality of the paper. It is also used in the manufacture of 2-furaldehyde and certain other bulk chemicals from agricultural waste products [3].

The amine-catalysed degradation is called nonenzymic browning or Maillard reaction [4]. It is very important in the processing and storage of food, being largely responsible for the colour, aroma and flavour of cooked food. The nutritional value may be reduced by the partial destruction of the essential amino acid lysine, but no toxic Maillard reaction products are believed to form during cooking under normal conditions. The Maillard reaction has also been observed in living organisms, including man. Thus, diabetic patients may develop cataracts, because the eye lens proteins are slowly attacked by glucose [5]. Their hemoglobin is modified in the same way.

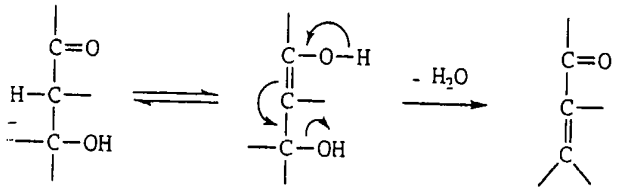
In the present investigation, carbohydrates are degraded in the absence of amines. The degradation of pentoses [6,7], hexoses [7-9], and hexuronic acids [6,10] in alkaline or acidic aqueous solution has been studied previously. The number of identified products has increased greatly in recent years, owing to the development of sophisticated techniques for separation and identification. Pentoses produce more colour than hexoses, and uronic acids cause more severe yellowing than aldoses.

Carbohydrate degradation is accompanied by dehydration and therefore results in increasingly unsaturated, partly aromatic products. Finally, an insoluble dark-brown polymer is obtained. The structure of this polymer and the mechanism of its formation are largely unknown, although phenolic and enolic compounds have been suggested as colour precursors [6]. However, the mechanism of the intial reaction steps in the degradation has been fairly well known for more than 20 years [11,12]. The degradation starts at the reducing terminal of a sugar. In oligo- and poly-saccharides, such terminals may be created gradually by cleavage of glycosidic bonds. This may occur through hydrolysis or "peeling" [2]. The reaction course may be affected considerably by the reaction time and temperature, pH, catalyst, and the nature and concentration of the reactants, but certain reaction steps are quite general. Some of those occurring in the absence of amines are shown in Scheme 1 [13].

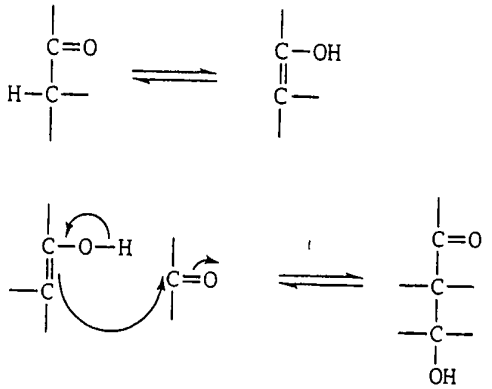
(i) Isomerization



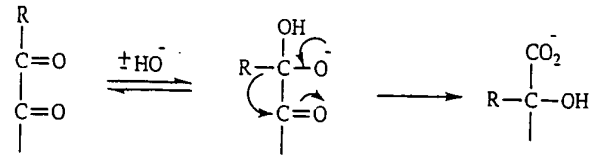
(ii) β-Elimination



(iii) Aldolization and retroaldolization



(iv) Benzilic acid rearrangement



Scheme 1. General reaction steps in sugar degradation.

Reactions (i)-(iii) in Scheme 1 may be catalysed through protonation of a reactant by acid or through proton removal from a reactant by base. Accordingly, reactions (i)-(iii) proceed via an enol in acid and via an enolate ion in alkali. These proton migrations are not shown in Scheme 1.

Reaction (i) may be exemplified by the isomerization of D-glucose to D-fructose and has now been known for a century [14]. Reaction (ii), i.e., β-elimination of a hydroxyl group, is a major cause of dehydration in sugar degradation. In retroaldolization, the carbon chain of the sugar is cleaved, resulting in fragmentation of the sugar. In aldolization, the fragments may recombine in the same or in a different way. Reaction (iv), i.e., the benzilic acid rearrangement, takes place in alkali only and gives rise to the so-called saccharinic acids [2,12].

In strongly acidic media, β-elimination is faster than other reactions. The sugar will therefore be dehydrated as soon as it has enolized, giving rise to relatively few products, sometimes in high yields. With increasing pH, tautomerization finally becomes faster than β-elimination, resulting in a more complex product mixture. In alkali, reactions (iii) and (iv) become important, resulting in a very complex product mixture through branching, cyclization, and extension or fragmentation of the carbon chain. In general, the degradation is much faster in basic than in acidic solution, because of the great catalytic effect of the hydroxide ion.

1.2. Starch/Glycogen-Metabolizing Enzymes

1.2.1. General Aspects

Starch is one of the most widely distributed naturally occurring organic compounds. It is a high-molecular polymer and represents an intricate arrangement of two polysaccharides, one being a branched fraction known as amylopectin and the other a linear fraction known as amylose. Each starch type contains these two fractions in a particular characteristic ratio. Amylose and amylopectin are both formed from D-glucose units. The linear polymer, amylose, is an unbranched molecule composed of α-1,4-D-glucose units, while the branched amylopectin is based on α-1,4- and α-1,6-linkages. The latter linkages are from the so-called branching points in the molecule [15].

Starch, glycogen and related oligosaccharides are of prime importance in the living process and life cycle of organisms. Starch may be decomposed by heating [16] or by enzymes. For many years, interest has centred on the enzymes responsible for the hydrolysis of starch as well as on those enzymes involved in the further metabolism of the hydrolysis products. Enzymes, promoting the hydrolysis of starch to reducing sugars, were detected over a century ago in a variety of biological materials and were intensively investigated [17].

The term starch-degrading enzymes usually designates a relatively wide range of enzyme activities which decrease the degree of polymerization of any oligo- or polyglucan predominately containing α-1,4-linked glucose units. Several types of such

enzymes are distinguished and listed below, together with the respective Enzyme Commission (EC) number [18] and the main reaction catalysed. The research interests have focused on the well-known starch hydrolases (amylases) and starch phosphorylases as well as the degradation products glucose and glucose-1-phosphate [18,19].

1.2.2. α-1,4-Glucan Phosphorylases (EC 2.4.1.1)

These phosphorylases have been found in chloroplasts of plants. Their pH optimum is 6.5 [15,18]. The enzymes transfer a glucosyl residue from the free non-reducing end of an amylose-like chain to orthophosphate, forming glucose-1-phosphate. Removal of glucosyl residues from the non-reducing end of the polyglucan chain continues until the glucan is converted to maltotetraose. In branched glucans, glucosyl removal continues until a limit dextrin is produced [15]. The reaction catalysed by the phosphorylases is reversible. Maltose phosphorylase is almost similar to the glucan phosphorylases, but its action is restricted to maltose as glucosyl donor and it has EC 2.1.1.8.

1.2.3. Amylases

Starch hydrolases are widely distributed in Nature, occurring in animals, plants, and micro-organisms. They hydrolyse α-1,4-linked oligo- and poly-glucans and are traditionally divided into three groups according to their action patterns [20].

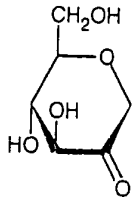
Endoamylases (α-amylases, EC 3.2.1.1). - α-Amylases are found in isolated chloroplasts. Their pH optima are between 4.75 and 6. α-Amylases cleave internal α-1,4-bonds of linear or branched glucans in a random manner, producing new reducing α-glucose units. The α-amylases cannot hydrolyse the α-1,6-linkages of amylopectin. Maltose is also resistant to α-amylases. The hydrolysis by α-amylases is hindered in the region of the α-1,6 branch points. The products of amylose hydrolysis are maltose and a small amount of D-glucose, while the hydrolysis of amylopectin yields D-glucose and a limit dextrin containing α-1,6- and some α-1,4-linkages.

Exoamylases (β-amylases, EC 3.2.1.2). - β–Amylases are located outside the chloroplast and possess a pH optimum of 4-5. Degradation of amylose by β-amylases starts from the non-reducing end of the glucan chains and proceeds by stepwise removal of maltose units. Amylopectin is hydrolysed like amylose beginning at the non-reducing ends, until the branch points are reached.

Debranching Enzymes (glucoamylases, EC 3.2.1.3). - Glucoamylases hydrolyse α-1,6-bonds in those glucans which contain both α-1,4- and α-1,6-bonds but have little effect on isomaltose and dextrans.

1.2.4. α-1,4-Glucan Lyase (EC 4.2.2.-)

The novel degrading enzyme α-1,4-glucan lyase has been isolated from the chloroplasts of the red algal cells of the red seaweeds *Gracilariopsis lemaneiformis* and *Gracilaria verrucosa* [21]. It degrades α-1,4-linked oligo- and poly-glucan chains to 1,5-anhydro-D-fructose (**1**).



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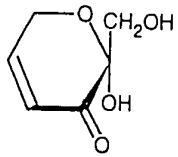


microthecin

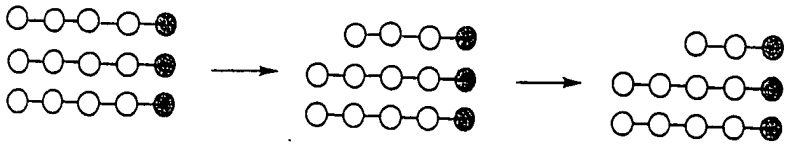
Compound **1** was first prepared in 1980 [22]. It was found in Nature six years later, when it was isolated from a new *Pseudomonas* strain growing on 1,5-anhydro-D-glucitol [23]. It has been found that **1** is a precursor of microthecin (one enantiomer shown) in morcels and other fungi [24] and that glycogen is the precursor of **1**. Subsequently, the presence of enzymes capable of converting glycogen to **1** was demonstrated. However, it was not clear whether the production of **1** from glycogen was catalysed by one or more enzymes. Due to the presence of other α-glucan-degrading enzymes in the enzyme preparation, an accurate characterization of the enzymes was not possible.

The chloroplastic location of the lyase contrasts with the fact that starch granules are located in the cytosol [21] and with the observation that this enzyme is able to hydrolyse both heat-denaturated starch and native starch granules. However, this may indicate the presence of a pool of soluble α-glucans, such as maltose and maltosaccharides, in the algal chloroplasts. Presently, the exact role of the α-1,4-glucan lyase inside the red algal chloroplast is unknown. However, no **1** was isolated from the algal extract, while a small amount of microthecin could be detected. The lack of **1** could be due to the activity of other enzymes able to convert **1**.

1.2.5. Mode of Action

In principle, the degradation of the polymer proceeds by one of three ways [15]. (i) After the initial cleavage of a polymer molecule, this is degraded completely by successive removal of small fragments before the enzyme initiates an attack on another polymer molecule (single chain attack, Fig. 1). (ii) The products may be released from the enzyme after the initial cleavage and for subsequent cleavages, the

Single chain attack



Multichain attack

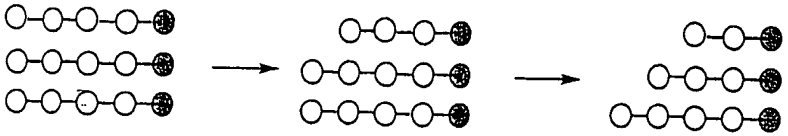


Fig. 1. The hydrolytic enzyme cleavage of a soluble polyglucan by single and multichain attacks.

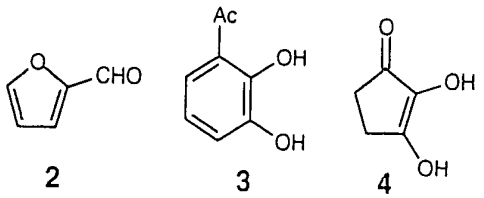
enzyme attacks other polymer molecules (or their fragments) in a random way (multichain attack). (iii) A process intermediate between single and multichain attack. The fragments are then attacked repetitively before the enzyme-substrate complex dissociates (multiple or repetitive attack).

Unlike the multichain attack, both the repetitive and the single chain attack result in the formation of low-molecular glucans in significant amounts, even in the initial period of amylose degradation.

1.3. Aim of the Thesis

The purpose of the present investigation was to obtain more information on the reaction mechanisms responsible for carbohydrate degradation by means of high-field ^{1}H and ^{13}C NMR spectroscopy, combined with isotopic tracer techniques. The following reactions were selected for study.

- The non-enzymatic degradation of pentoses in slightly acidic, aqueous solution is treated in Chapter 2. The formation of 2-furaldehyde (2) [I], formic acid [I], 2,3-dihydroxyacetophenone (3) [II], and reductic acid (2,3-dihydroxy-2-cyclopentenone, 4) [III] has been studied.
- The degradation of starch to 1,5-anhydro-D-fructose (1) by the new enzyme α-1,4-glucan lyase [IV] is treated in Chapter 3. The substrate specificity of the lyase has also been explored.
- Some further reactions of 1 in alkaline, aqueous solution [V] are treated in Chapter 4.

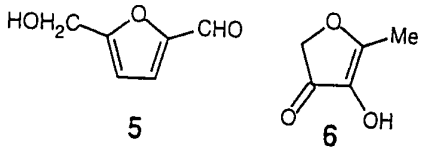


2 3 4

2. DEGRADATION OF PENTOSES

2.1. Formation of 2-Furaldehyde

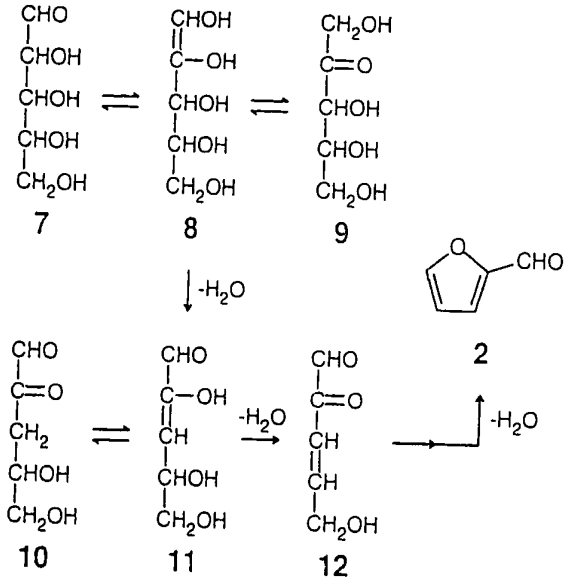
As mentioned above, high yields of degradation products may be obtained from carbohydrates in strong acid. These products are furan derivatives. 5-(Hydroxymethyl)-2-furaldehyde (5) is the main reaction product from hexoses and hexose derivatives, notably from sucrose [25], while pentoses and hexuronic acids give 2-furaldehyde (2). Thus, 2 has been produced from bran, corncobs, and other waste materials on an industrial scale [3]. The aldehydes 2 and 5 may polymerize to brown compounds, but 2 may be obtained in high yield if it is removed by distillation as soon as it is formed. In weaker acid, the furanone 6 may be the main product. It has been obtained from xylose in 20% yield [26] and from ribose-5-phosphate in 47% yield [27].



5 6

The mechanism of pentose dehydration to 2-furaldehyde (2) has been a matter of study for several years. The accepted pathway of the reaction in strongly acidic media was initially suggested by Isbell [28] and has been modified slightly by later work [12]. It is shown in Scheme 2, where the stereochemistry is neglected in order to make the scheme valid for all pentoses. In this mechanism, the pentose is assumed to react exclusively through its acyclic form (7 or 9). This is slowly converted into its 1,2-enediol 8, followed by dehydration to the enolic form 11 of the 3-deoxypentosulose 10 through β-elimination. The enol 11 is further dehydrated to the 3,4-dideoxypent-3-enos-2-ulose 12 prior to cyclization to 2. The mechanism implies incorporation of hydrogen from the solvent into the furaldehyde at positions α and 3. However, when xylose was dehydrated in tritiated strong acid, the resulting 2 contained very little tritium [29].

This observation has been rationalized in several ways. According to one proposal, 7 and 9 are not interconverted via the enediol 8 but directly through an intramolecular 1,2-shift of hydrogen [30]. From time to time [12,25,31], reaction routes from sugars to 2 or 5 via cyclic intermediates have been suggested. A third explanation is that in strong acid, the enols 8 and 11 are dehydrated much faster than they tautomerize [12]. In order to test this hypothesis, it would be desirable to study the formation of 2 or 5 in weaker acid, where β-elimination is slower than tautomerization. Apparently, this has been done only in the presence of various amines [32]. Partial exchange of H-α and H-3 in the resulting 5 was indeed observed, but this was ascribed to the amine catalyst rather than to the weaker acid (deuterated 2 M acetic acid).



Scheme 2. Classical route to 2-furaldehyde (2) from a pentose (7 or 9).

For these reasons, the formation of 2 from pentoses in weak acid and in the absence of amines has now been investigated [1]. The disappearance of the pentose and the formation of 2 in 0.5 M deuterated sodium phosphate buffer were followed by ^{1}H NMR spectroscopy at 96°C and pD 1.5, 3.0, and 4.5. With increasing pD, the yield of 2 decreased, due to the formation of other products [6,33]. These were indicated by a strong colour of the solution and by formation of a dark precipitate. The yields of 2 calculated on consumed pentose at the end of the degradation experiments are shown in Fig. 2.

Increasing pD strongly accelerated the degradation of the aldoses but slightly retarded that of the ketoses. As expected from Scheme 2, the relative degradation rate of each pentose was governed by the abundance of its acyclic form at equilibrium, which in turn is inversely related to the stability of its ring forms. Thus, the *all-cis*-configuration of α-D-ribopyranose leads to a *syn*-1,3-diaxial interaction in both the chair conformers (Scheme 3), resulting in a lower proportion of this isomer and a higher proportion of all other isomers, including the aldehydo isomer, compared to the other aldopentoses [34]. This may explain why ribose was degraded faster than

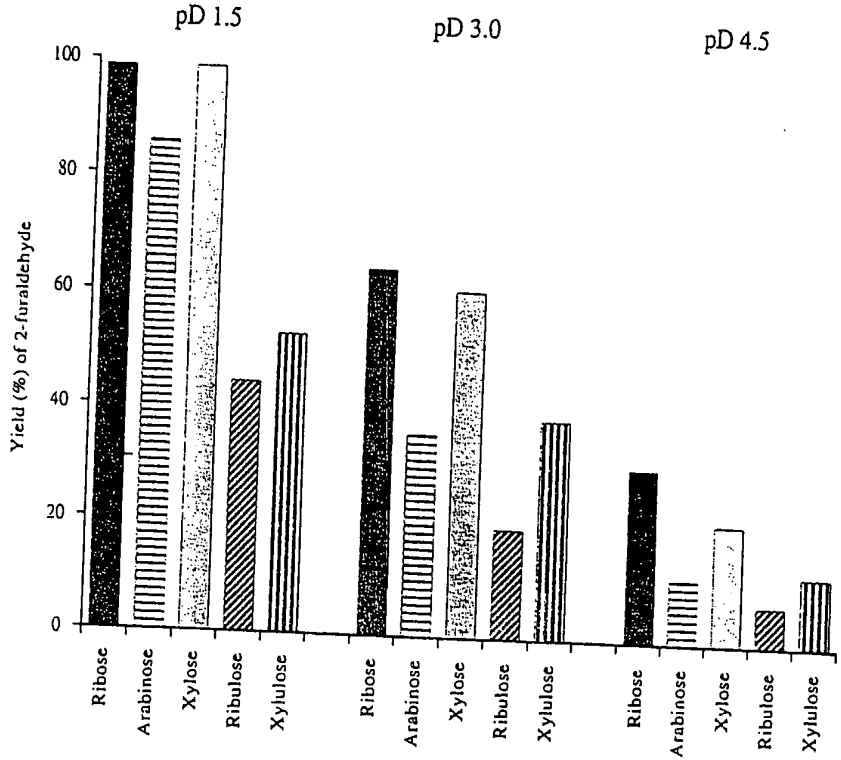
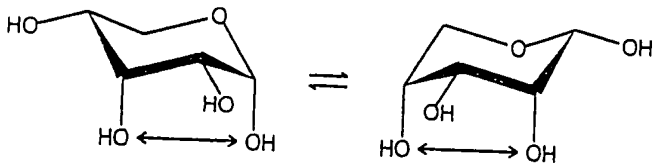


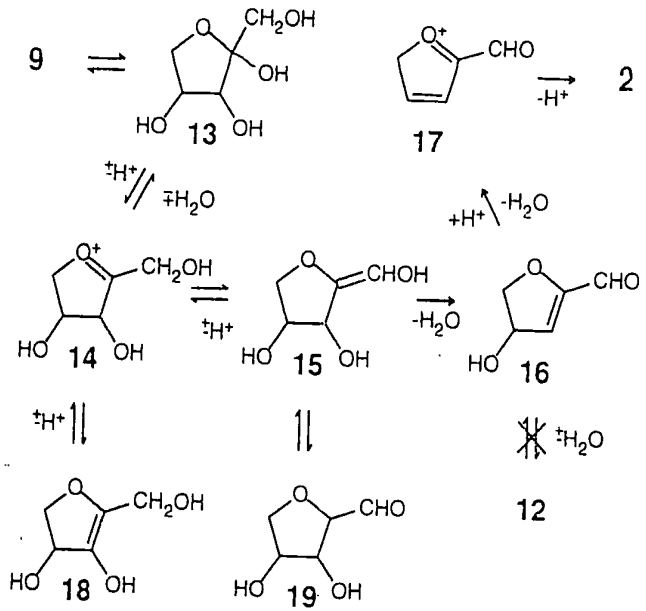
Fig. 2. Yields of 2-furaldehyde (2) calculated on consumed aldopentose after 72 h and on consumed ketopentose after 32 h.

arabinose and xylose. Since no pyranose forms of the ketopentoses exist, these contain an exceptionally high proportion of keto form [35]. Accordingly, the ketopentoses were degraded much faster than even ribose. This was probably the reason why only minor amounts of pentuloses were observed in the degradation of the aldoses.

The ^{1}H NMR spectra showed that H-α and H-3 in 2 had largely been replaced by deuterium. The higher the pD, the more deuterium was incorporated and at pD 4.5, the exchange of H-3 was virtually complete. Hence, the enol 11 tautomerized faster to 10 than it was dehydrated to 12, unlike its behaviour in stronger acid, and the 1,2-enediol 8 tautomerized to a ketose 9 faster than it was dehydrated to 11. No hydro-



Scheme 3. 1,3-Diaxial interactions in the α-D-ribopyranoses.



Scheme 4. Alternative route from pentoses to 2-furaldehyde via cyclic intermediates.

gen exchange was observed in the aldose 7, indicating that 7 was converted irreversibly into the enediol 8.

An alternative route to 2 is shown in Scheme 4. It is analogous to previously [12,25] suggested routes from fructose to 5 in strong aqueous acid and involves only cyclic intermediates, starting with the ketofuranose 13. In order to explain the observed deuterium incorporation into 2, we must assume that the enol 15 has time to equilibrate with 18 via 14, before it is dehydrated to 2 via 16 and 17. If so, the 2,5-anhydropentose 19 should also form, but this was never observed. For this reason, the route in Scheme 4 appears less probable than that in Scheme 2. For the same reason, a route from xylose via 19 to 2 [31] seems unlikely.

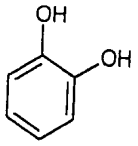
2.2. Formation of Formic Acid

The first degradation of a carbohydrate by acid (sucrose by sulfuric acid) to formic acid was reported more than 150 years ago [36]. From hexoses or hexose derivatives, formic acid is formed via 5 along with levulinic acid [12,25]. The formation of formic acid from xylose has been reported [31], but the reaction mechanism has not yet been elucidated. In all the pentose degradation experiments reported in Paper [I] and discussed in the preceding section, formic acid was formed in low yield along with 2-furaldehyde (2), but no formic acid was formed from 2 under any of the con-

ditions used. To get more information about the reaction mechanism, some 1-^{13}C- or 5-^{13}C-substituted pentoses were treated under the same conditions. As evident from its ^{13}C content, the resulting formic acid was derived exclusively from the terminal pentose carbons, C-1 being somewhat more important than C-5. Its formyl hydrogen had been partly replaced by deuterium, but it is hard to draw further conclusions about the mechanism from this observation.

2.3. Formation of 2,3-Dihydroxyacetophenone

Disregarding the chromone **24** [37], the first phenolic products from carbohydrates were identified in 1970 [38]. In this and later studies on degradation and yellowing of cellulosic products during storage and heat treatment, catechol (**20**) and a number of its derivatives were obtained from various sugars. Under slightly acidic conditions, **3**, **20-22**, and **24-26** were obtained from pentoses or hexuronic acids [6,39], while **23** and **28** were obtained from hexoses [9]. The total yields of phenolic compounds isolated from the degradation of the mentioned sugars were 0.3, 1 and 7%, respectively [40]. These reactive compounds could have formed in much higher yields, since they are readily transformed into coloured polymers.



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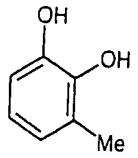

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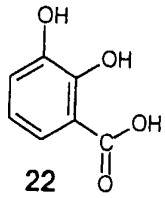

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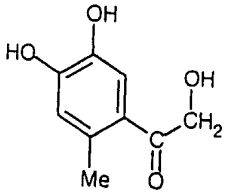

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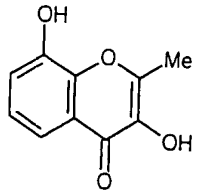

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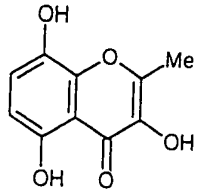

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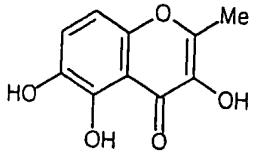

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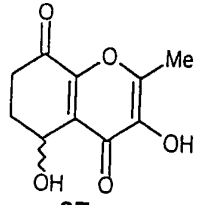

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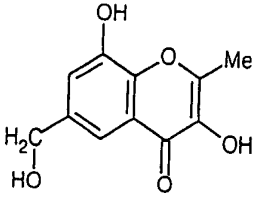

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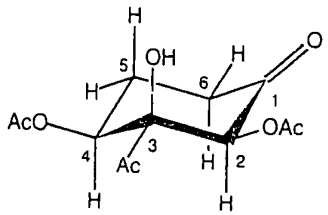
The chromone **24** may be regarded as an anhydro-dimer of a pentose. A clue to the mechanism of its formation was offered by the trapping of its alicyclic precursor **27** [6,38] and soon afterwards, the reaction route shown in Scheme 5 was proposed [41]. In this route, which was later supported by isotopic tracer techniques [42], one pentose molecule is assumed to form the 2-furaldehyde precursor **12** according to Scheme 2 above. Enolization of **12** then gives the hypothetical species **32**. The other pentose molecule is supposed to react via the ketose **9** and the 2,3-enediol **29**, which is dehydrated through β-elimination to the α-diketone **30**. This is believed to tautomerize to the β-diketone **33**, which is the precursor of the furanone **6**. Finally, the pyrone **27** is thought to form by Michael addition of **33** to **32**, followed by cyclization through aldolization. The further dehydration of **27** to **24** is fairly trivial.

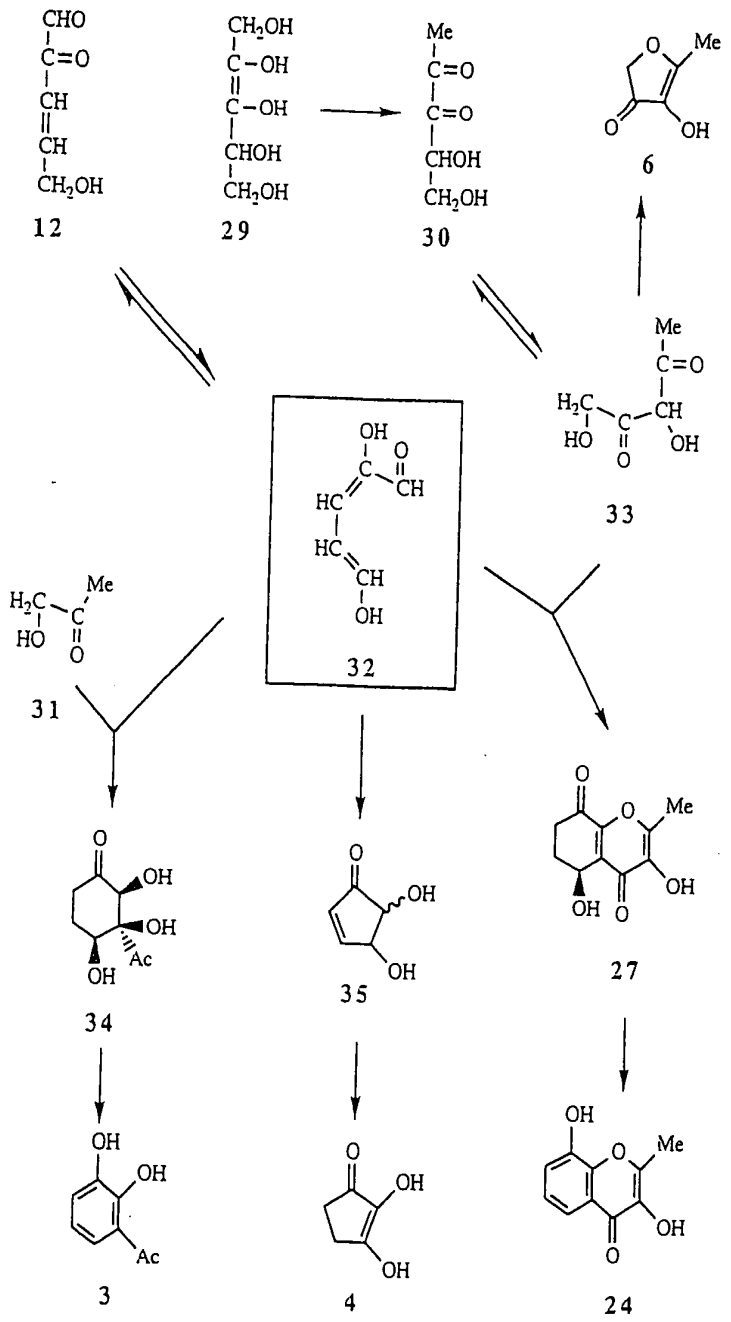
An alicyclic precursor of 2,3-dihydroxyacetophenone (**3**) has also been trapped and suggested to be an isomer of **34** [6,38]. However, structure **34** was not taken into consideration. Moreover, the formation and further dehydration of the precursor were explained more easily by formula **34** than by any isomeric structure.

This problem has now been solved by high-field ^{1}H NMR spectroscopy in favour of structure **34** [II]. For that purpose, the diacetate **34a** (one enantiomer shown) of the precursor was prepared and its 400 MHz ^{1}H NMR spectrum analysed. The four methylene protons were assigned owing to the strong coupling between axial and geminal protons and to the influence of the adjacent carbonyl group. The other protons were assigned mainly from NOE difference spectra.

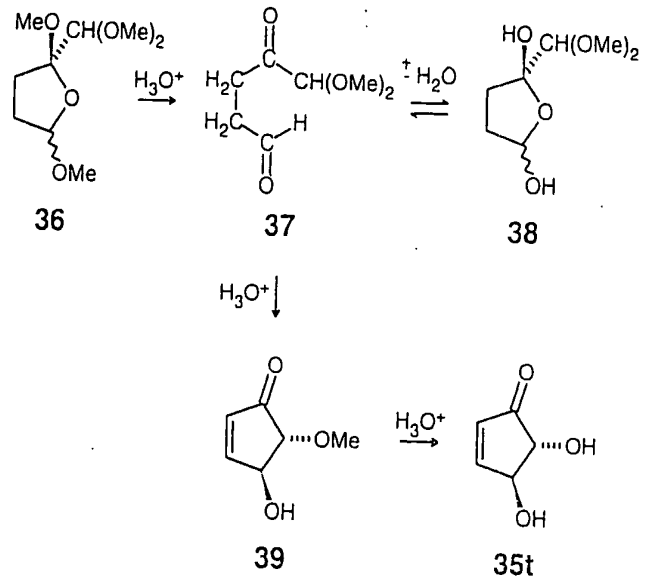


34a

Acetol (**31**, Scheme 5) is a common sugar degradation product [43]. It may be formed by acid cleavage (retro-Claisen condensation) of β-dicarbonyl intermediates, such as **33**. As indicated in Scheme 5, the formation of **34** could be analogous to that of **27**, i.e., Michael addition of acetol (**31**) to **32**, followed by cyclization through aldolization. If so, the hypothetical species **32** may be a general precursor of phenolic products in the degradation of pentoses or hexuronic acids.



Scheme 5. Possible routes to some enolic and phenolic degradation products of pentoses. For compounds 27 and 34, only one enantiomer is shown.



Scheme 6. Hydrolysis of the acetal 36 to the reductic acid isomer 35t.

2.4. Formation of Reductic Acid

In order to confirm the route to 34 and 3 in Scheme 5, a stable source of 32 was needed for reaction with 31. For that purpose, the acetal 36 (Scheme 6) was prepared from 2-furaldehyde dimethyl acetal via the 2,5-dihydro derivative [44]. The main product obtained on hydrolysis of compound 36 in weak acid was the oxopentanal 37 (isolated in about 40% yield), which in water mainly exists as its diastereometric hydrates 38 [III]. However, when the hydrolysis was repeated in the presence of acetol (31), this did not react with 37, and neither 34 nor 3 was obtained.

The main reason for this failure became apparent on further reaction of 37 in dilute acid, which yielded about 10% each of compounds 39 and 35t (the *trans* isomer of 35). This reaction apparently involved intramolecular aldolization of an enol form related to 32. One enantiomer of the 5-(hydroxymethyl) isomer of 35t was recently obtained by enzymatic degradation of 1,5-D-anhydrofructose (1) [45].

The product 35t is an isomer of reductic acid (4), which has been obtained by acid treatment of keto-glycosides [46] or sugars [6]. As indicated in Scheme 5, reductic acid is believed [12,28] to form via 32 and 35. However, the cyclopentenone 35t did not form reductic acid 4 on treatment with dilute acid or base, an observation that is inconsistent with the previously [28] suggested tautomerization mechanism.

3. DEGRADATION OF STARCH BY α-1,4-GLUCAN LYASE

3.1. Enzyme purification and characterization

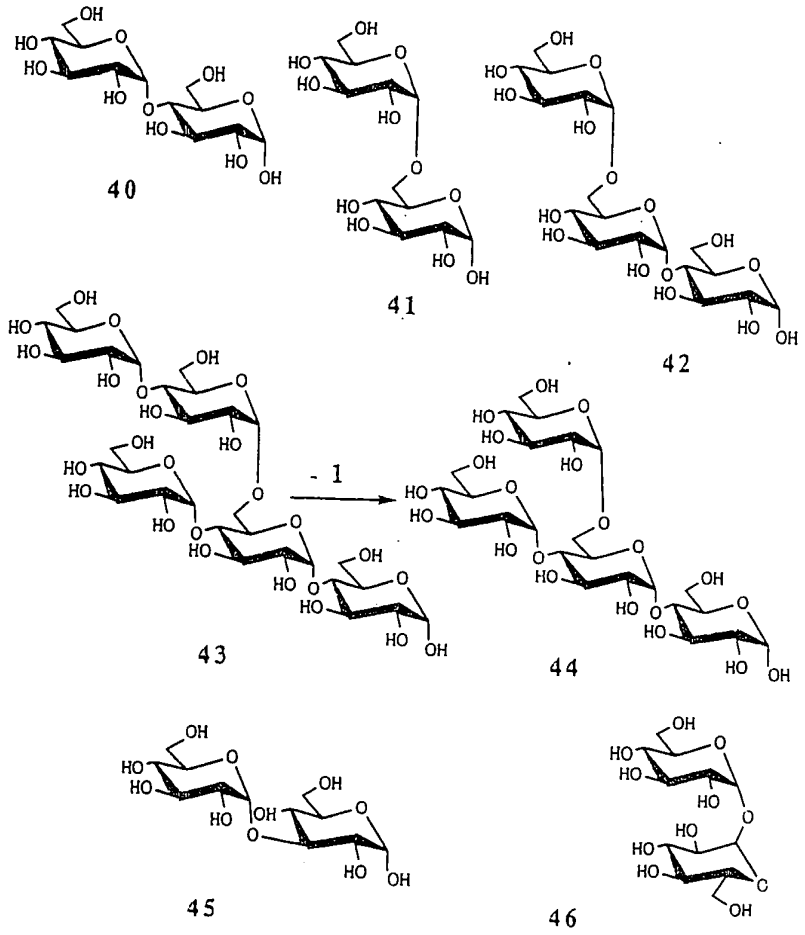
The α-1,4-glucan lyase was purified by using a starch column, followed by ion exchange chromatography on Q-Sepharose and filtration on Sephacryl S-200 [IV]. The method developed for the purification of the enzyme was cheap with high purification factor and recovery rate. The purified enzyme has a molecular mass of ca. 116,500 Da. Partial amino acid sequences of the lyase with a total length of 100 amino acid residues were obtained. Amino acid composition analysis of the enzyme showed high amounts of Asp/Asn, Gly and Glu/Gln. The isoelectric point of the enzyme was 3.9, as revealed by isoelectrofocusing. α-1,4-Glucan lyase showed a wide pH optimum range from pH 2.5 to 7.0 for maltose and from pH 3.5 to 7.5 for amylopectin, wider than that of any other α-glucan-degrading enzyme [47]. The optimum temperature for the algal lyase was 50°C with maltose or amylopectin as substrate.

3.2. Substrate specificity of the enzyme

The lyase catalyses the degradation of maltose (**40**, Scheme 7), malto-oligosaccharides, starch and glycogen to 1,5-anhydro-D-fructose (**1**). This is the major difference between α-1,4-glucan lyase and the classical starch-degrading enzymes, such as amylases and phosphorylases, which degrade starch and glycogen to glucose, maltosaccharides, or glucose-1-phosphate.

The algal lyase readily cleaved α-1,4-glucosidic bonds in maltosaccharides and branched α-glucans [IV]. It was, however, not able to cleave the α-1,4-glucosidic bond at position 4 of a 4,6-branched residue, as in the tetrasaccharide 6^2-α-D-glucosylmaltotriose (**44**) obtained from cleavage of the pentasaccharide 6^2-α-D-maltosylmaltotriose (**43**) with the lyase (Scheme 7). The ability of the lyase to cleave α-1,6- or α-1,3-glucosidic linkages is very limited. Thus, the lyase was not able to degrade isomaltose (**41**), while it cleaved the α-1,6-bond in panose (**42**) and the α-1,3-bond in nigerose (**45**) at a very slow rate. The lyase was not able to cleave α,α-trehalose (**46**), cellubiose (Glc*p*β1 → 4Glc), sucrose (Glc*p*α1 → 2β-Fru*f*), melibiose (Gal*p*α1 → 6Glc), or lactose (Gal*p*β1 → 4Glc).

No activity was detected towards the α-1,4-linkage of other α-glycosides, such as *p*-nitrophenyl α-D-mannoside or α-D-galactoside (with an axial hydroxyl at C-2 or C-4). The inability of the lyase to degrade cyclohexaamylose indicates that it is an exo-lyase and has little or no endo-cleavage activity. The lyase was not able to dehydrate monosaccharides, such as glucose, galactose and mannose.



Scheme 7. Oligosaccharides with α-D-1,4-; α-D-1,6-; α-D-1,3-; and α-D-1,1-linkages: **40**, maltose; **41**, isomaltose; **42**, panose; **43**, 6^2-α-D-maltosylmaltotriose; **44**, 6^2-α-D-glucosylmaltotriose; **45**, nigerose; **46**, α,α-trehalose.

3.3. Mechanism for cleavage of the glucosidic linkage

α-1,4-Glucan lyase degrades soluble starch amylose via multichain attack (Fig. 1). After the initial lytic cleavage of an α-1,4-linkage, the maltosaccharide product is released from the enzyme and for subsequent cleavage, the enzyme attacks all the substrate molecules in a random fashion. After incubation of maltoheptaose with the lyase for only 2 min [IV], more than one third of the maltoheptaose had been converted into maltohexaose, a small amount of maltopentaose, and 1. With increased

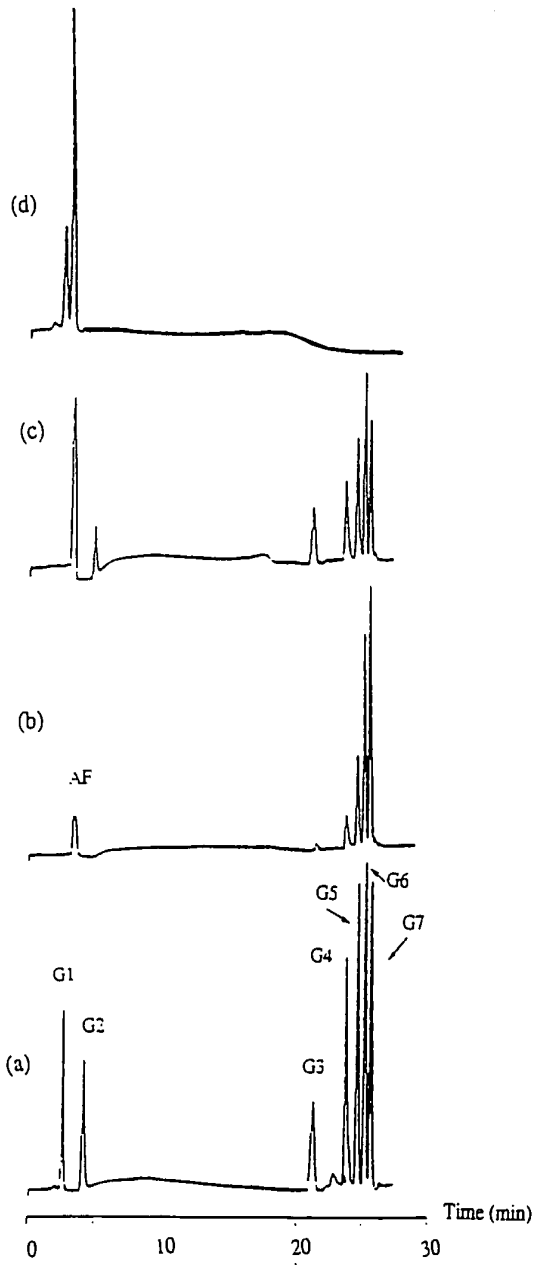
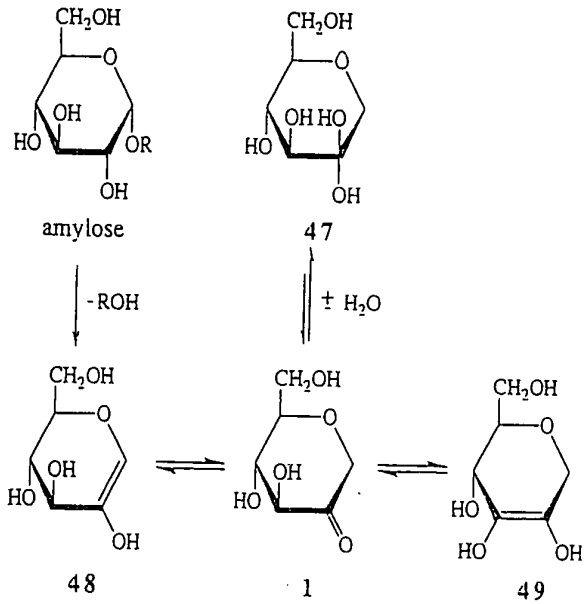


Fig. 3. The time course of the lyase degradation of maltoheptaose as monitored by HPLC, (a) standard mixture of sugars G1-G7, (b) lyase reaction with maltheptaose after 20 min, (c) after 1 h, and (d) after 5 h. G1-G7 represent glucose, maltose, maltotriose, etc.



Scheme 8. The four forms of 1,5-anhydro-D-fructose, 1 and 47-49.

incubation time, lower maltosaccharides appeared and increased in amount. The degradation of maltoheptaose by lyase action was monitored by HPLC on a Dionex system (Fig. 3), TLC, and ^{1}H NMR spectroscopy. The whole reaction was complete after incubation for 5 h, and the product ratio of 1 to glucose reached a value of 6:1. In the lyase reaction with maltose, this ratio was 1:1.

Amylose, (1-^{2}H)-, and (2-^{2}H)-amylose were treated separately with the lyase in H_2O or D_2O. The ^{1}H NMR spectra of the reaction mixtures showed that the lyase cleaved α-1,4-linkages from the non-reducing end of an α-1,4-glucan. The most reasonable reaction path seemed to be an elimination, creating a double bond between C-1 and C-2 of the terminal glucose unit (Scheme 8). The resulting enol form 48 of 1,5-anhydro-D-fructose would then react via the keto form 1 to the very stable hydrate 47 (see Chapter 4).

Incorporation of deuterium at C-1 was indeed observed in the degradation of amylose in D_2O, confirming the intermediacy of the enol 48. However, the deuterium content at C-1 of 47 was less than 50 %, and some deuterium also appeared at C-3, implying that the 2,3-enediol 49 was also involved. It should be noted that the equilibria in Scheme 8 are shifted strongly towards 47. Thus, the ^{1}H NMR spectrum of 47 revealed no hydrogen exchange with the solvent, even after one week. Hence, the lyase degradation reaction is certainly more complex than indicated by Scheme 8. Thus, the additional hydrogen at C-1 of 1 is derived not only from the aqueous reaction medium but also from H-1, H-2, and H-3 of the glucose unit.

4. DEGRADATION OF 1,5-ANHYDRO-D-FRUCTOSE

4.1. Previous work

Turning from the action of acid on reducing sugars to that of alkali, a much more complicated mechanism is encountered. The structural changes typically induced by the alkali might be divided into two classes: (i) those involving scission of the molecule into smaller fragments and (ii) those which involve rearrangement. The first class includes the formation of simple acids, such as formic, acetic, lactic, and hydroxybutyric acids, and the second class the formation of iso- and meta-saccharinic acids. It is this second class of reactions which has been important in the present study [V] on the degradation of 1,5-anhydro-D-fructose (1).

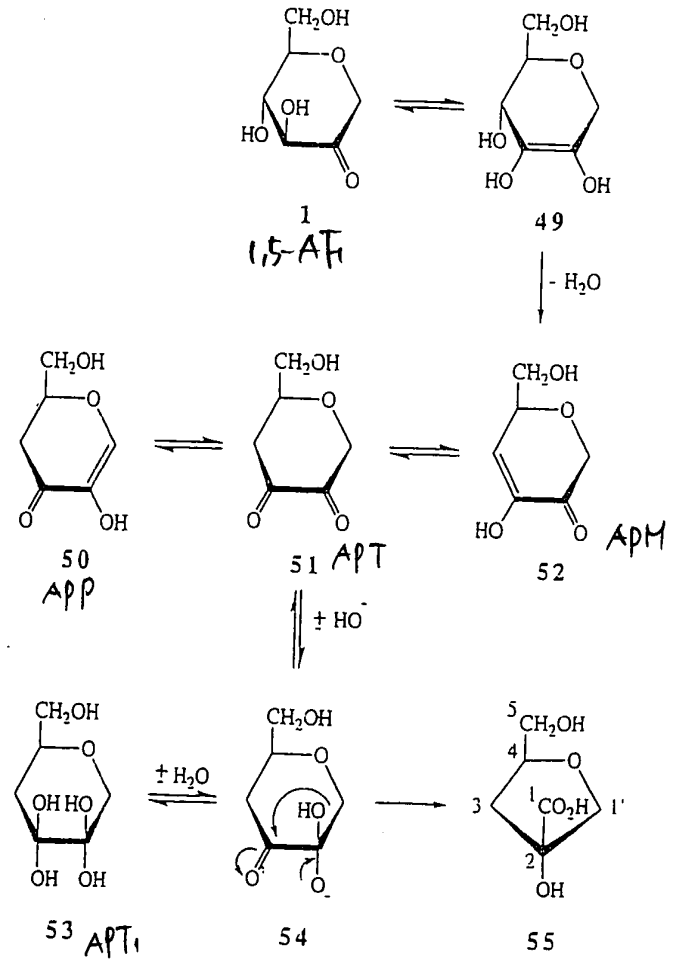
The chemistry of the so-called saccharinic acids has been reviewed [12,48]. The mechanism of formation proposed by Isbell [49] has been generally accepted. It involves formation of an enediol followed by β-elimination. The next step requires tautomerization to an α-dicarbonyl intermediate that can undergo benzilic acid rearrangement. The initial two steps are the same as those noted for acidic degradation, as exemplified by the formation of the pentosulose **10** from a pentose (**7** or **9**) according to Scheme 2. The differences between the acid- and base-catalysed reactions obviously lie in the final two steps, where tautomerization to the dicarbonyl intermediate is very rapid under basic condition. In acid, further dehydration, if possible, occurs rapidly, before equilibrium of the enediol with the dicarbonyl compound has been established.

4.2. Present work

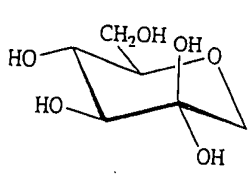
The reaction mechanism for the degradation of **1** in aqueous alkali [V] was similar to that for the base-catalysed degradation of reducing sugars. The probable reaction route is shown in Scheme 9. Actually, the reaction proceeds through the anions, but this has been indicated only for the species **54** undergoing the benzilic rearrangement, for easier comparison with reaction (iv) in Scheme 1. After reaction for 30 min in 0.5-1 M aqueous NaOH at 25°C, the saccharinic acid **55** was obtained as the final product on acidification. The determination of its structure, 1',4-anhydro-3-deoxy-2-*C*-(hydroxymethyl)pentonic acid, with ^{1}H and ^{13}C NMR spectroscopy was straightforward, and the D-*threo*-configuration was established from NOE measurements on the tri-*O*-methyl derivative. The same acid or its *erythro*-isomer has been obtained by alkaline degradation of cellulose [50].

In order to confirm the route in Scheme 9, the degradation was repeated in more dilute alkali and interrupted after 5 min. After neutralization, the reaction mixture was separated by preparative HPLC into two fractions. As shown by the NMR spectra, one of the fractions only contained the enol **50**, while the other was a mixture of the enol **52** and a derivative of the diketo form **51**. This derivative was either the dihydrate **53** or a bicyclic pyranose or furanose form (**57** or **58**).

The keto forms **1** and **51** were ruled out by the ^{13}C NMR spectra, which showed no signal above δ 100. The bicyclic pyranose form **56** was proposed [24] for 1,5-anhydro-D-fructose in aqueous solution, but the deuterium-induced ^{13}C shifts [51] clearly showed the hydroxyl group at C-6 to be free [V]. The hydrate form **47** was further confirmed by the FAB-mass spectrum, which showed $[M + Na]^+$ at *m/z* 203.



Scheme 9. Base-catalysed degradation of 1,5-anhydro-D-fructose (**1**) to the saccharinic acid **55**.



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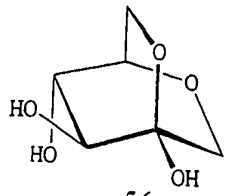

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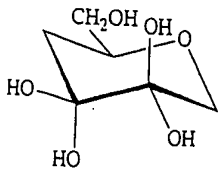

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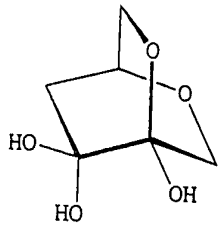

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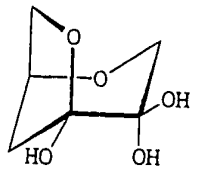

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So far, an unambiguous decision between formulas **53**, **57**, and **58** has not been made. The $^1H,^1H$ coupling constants $J_{5,6a}$ and $J_{5,6b}$ were 2.4 and 7.1 Hz. The difference between them seems to large to fit the bicyclic formulas **57** and **58**. On the other hand is formula **53** most unusual, although the chair conformation shown should be virtually strainless. The only α-diketones known to occur predominantly as dihydrates appear to be dioxosuccinic acid and its derivatives [52].

If **51** rearranges to **55** much faster than it enolizes to **52**, one of the hydrogens at C-3 in **55** must be derived from H-4 in **1** and the other from the solvent. Since **52** should be protonated preferentially from the least hindered side of the ring, the solvent-derived hydrogen at C-3 is expected to be *trans* to the carboxyl and hydroxymethyl groups (i.e., *pro-S*) in most molecules of **55**. If **51** rearranges slowly enough to permit reversible enolization to **52**, the contribution from the solvent to the hydrogens at C-3 in **55** will, of course, exceed 50%.

This is obviously the case, as shown by repeating the degradation of **1** in deuterated alkali. The H-3 signals in the ^1H NMR spectrum of the resulting **55** are shown in Fig. 4b and show nearly complete deuterium incorporation. For comparison, the same signals from ordinary **55** are shown in Fig. 4a. Moreover, the signal at δ 2.04 in Fig. 4b was substantially more intense than that at δ 2.14. According to NOE measurements, the latter signal corresponded to the *pro-S*-hydrogen. The preferential deuterium incorporation at this position had thus been confirmed.

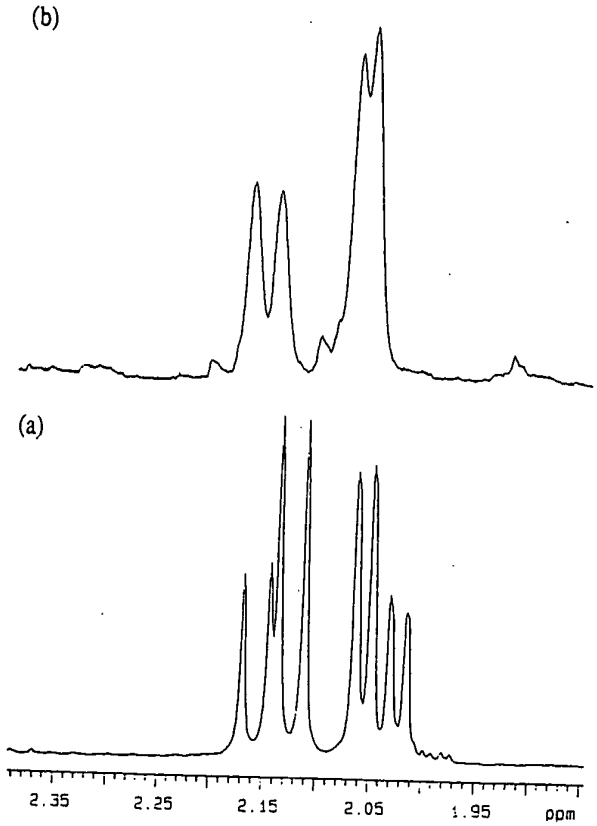
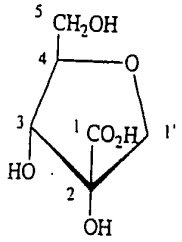


Fig. 4. H-3 signals in the ^1H NMR spectrum of the saccharinic acid **55**, obtained from 1,5-anhydro-D-fructose (**1**) in (a) NaOH-H_2O, (b) NaOD-D_2O.

Degradation of **1** substituted with ^{13}C in position 1 or 2 showed that C-2 of **55** originated from C-1 of **1** and that the carboxylic carbon (C-1) of **55** was derived from C-2 of **1**. Hence, the benzilic rearrangement had taken place exclusively as shown by formula **54** in Scheme 9, i.e., by cleavage of the bond between C-1 and C-2. The reason could be that the ring oxygen promotes the migration of C-1, since the migrating carbon formally moves as an anion. An alternative explanation is that the anion of **57** is rearranging rather than **54**.

To test its reducing capability, **1** was degraded in alkali as before but in the presence of 3,5-dinitrosalicylic acid (DNS). After 10 min at 25°C, followed by acidification, the DNS had been reduced to aminonitrosalicylic acid, and all **1** had been converted into an acid. This was shown by ^1H and ^{13}C NMR spectroscopy to be a hydroxyl derivative of **58**, viz., 1',4-anhydro-2-*C*-(hydroxymethyl)-D-arabonic acid (**59**). Compounds **50**, **51**, and **55** were shown not to be intermediates in this reaction.



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The configuration of **59** was established from NOE measurements on its tetra-*O*-methyl derivative. It was confirmed by the size (8.0 Hz) of the $^1H,^1H$ coupling constant $J_{3,4}$, indicating that H-3 and H-4 are *trans*-related.

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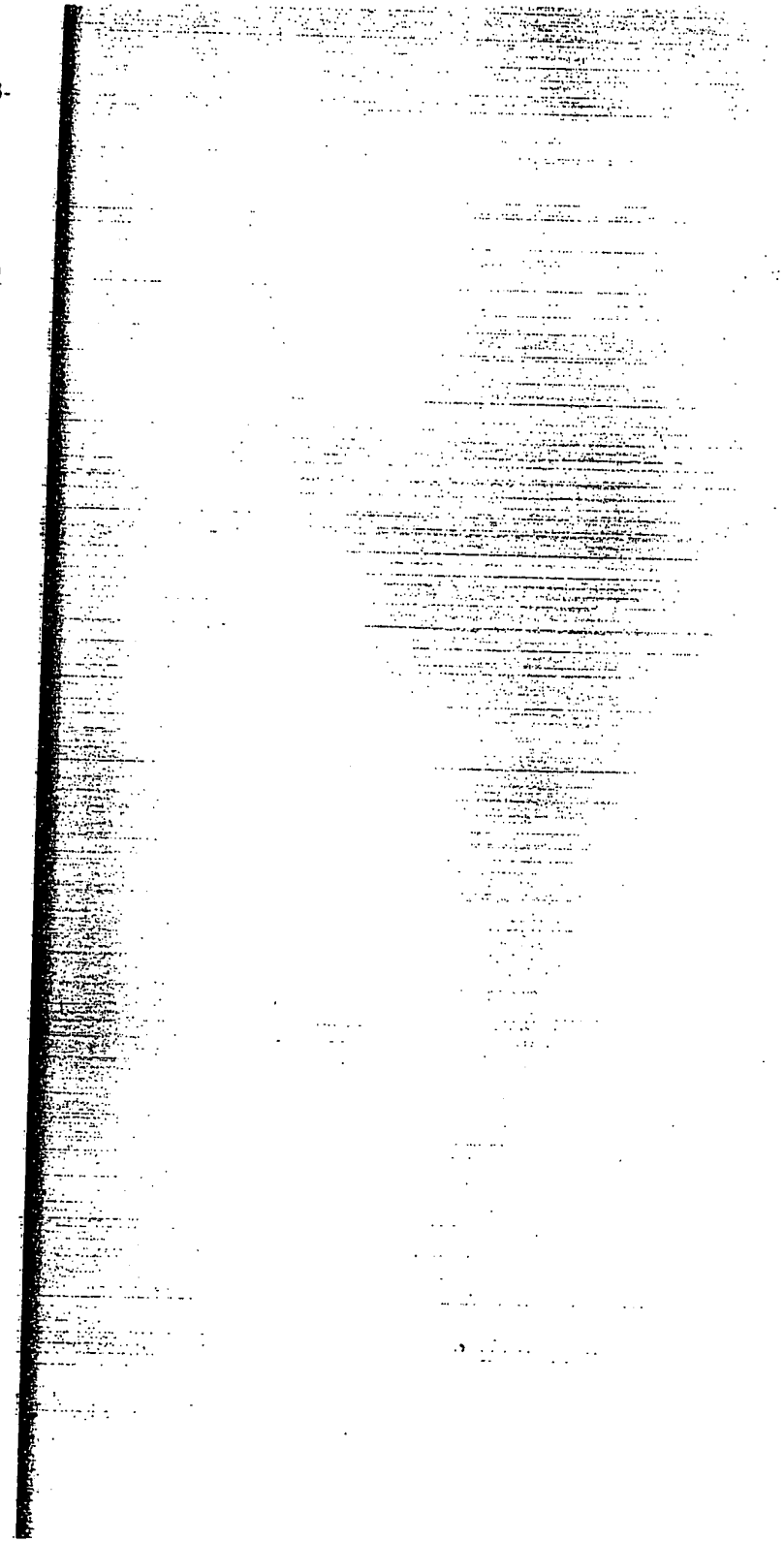

The formation of 2-furaldehyde and formic acid from pentoses in slightly acidic deuterium oxide studied by ^{1}H NMR spectroscopy

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Abstract

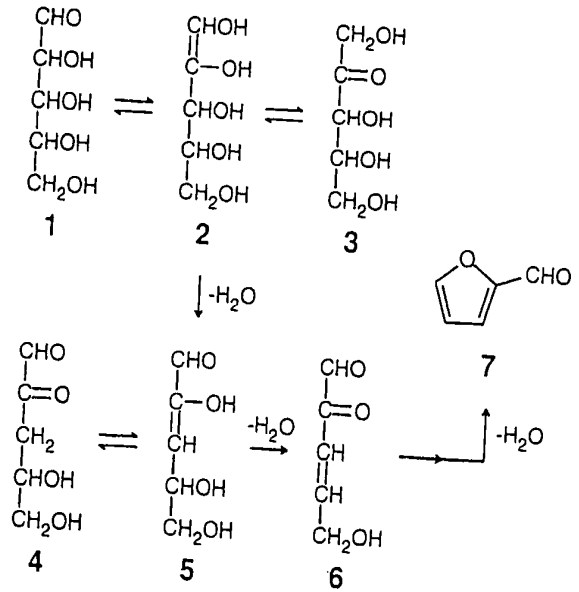
The title reaction at 96°C and pD 1.5, 3.0, or 4.5 was followed by ^{1}H NMR spectroscopy. The rate of pentose degradation increased in the order: arabinose ≈ xylose < ribose < 2-pentuloses. At pD 1.5, the rate of 2-furaldehyde (7) formation increased in the same order. Increasing pD strongly accelerated the degradation of the aldoses but slightly retarded that of the ketoses. Increasing pD also retarded the formation of 7, particularly from the ketoses, and increased its deuterium content at H-α (from 8-25 to 50-83 atom %) and H-3 (from 79-100 to 100 atom %). This is explained by assuming that 7 had formed mainly via acyclic intermediates, with reversible formation of a 3-deoxypentosulose. The formation of formic acid was slow and did not proceed via 7. As evident from experiments with 1- or 5-^{13}C-substituted aldopentoses, the formic acid was derived exclusively from the terminal pentose carbons, C-1 being somewhat more important than C-5.

Keywords: Formic acid; 2-Furaldehyde; Pentose

1. Introduction

Dehydration reactions of carbohydrates are important in pulping [1], in cooking (Maillard reaction) [2], and in the manufacture of certain bulk chemicals. Thus, 2-furaldehyde is obtained by the acid-catalysed dehydration of pentoses and hexuronic acids, present in the polysaccharides of various agricultural waste products [3]. The early literature on such reactions in aqueous media has been reviewed [4].

* Corresponding author.



Scheme 1. Classical route from pentoses to 2-furaldehyde via acyclic intermediates.

The first generally accepted reaction route [4] from an aldopentose (1) or a ketopentose (3) to 2-furaldehyde (7) is shown in Scheme 1 and proceeds via the intermediates 2, 5, and 6. Until fairly recently, sugars were believed to react exclusively through their acyclic forms. The more abundant cyclic forms of 1 and 3 are therefore ignored in Scheme 1. For further simplicity, the stereochemistry is also neglected.

When D-xylose was dehydrated in tritiated 3 M sulfuric acid, the main product 7 contained very little tritium [5]. This was rationalized by assuming that the enols 2 and 5 are dehydrated much faster than they tautomerize. An intramolecular hydride transfer from C-2 to C-1 in the xylose was proposed [6] as an alternative explanation for the absence of tritium at C-α of 7. More recently, an entirely new reaction route from D-xylose to 7 in strong aqueous acid was suggested [7]. This route involves only cyclic intermediates. The formation of formic acid along with 7 was observed but apparently not explained [7].

In weaker aqueous acid, D-xylose has been degraded into catechols and other compounds, 7 being only a minor product [8]. In aqueous alkali, no 7 was formed, and phenolic products predominated [9]. We have now reinvestigated the transformation of D-xylose and other pentoses into 7 and formic acid in slightly acidic deuterium oxide, using ^{1}H NMR spectroscopy.

2. Results

Three 0.5 M sodium phosphate buffers in deuterium oxide were prepared and adjusted to pD 1.5, 3.0, and 4.5 [1]. Each buffer contained 0.1% (w/v) of pivalic acid, to be used as an internal ^{1}H NMR standard. The degradation of the following D-pentoses was investigated: arabinose, ribose, ribulose (*erythro*-2-pentulose), xylose, and xylulose (*threo*-2-pentulose). A 2.5% (w/v) solution of each pentose in each buffer was heated at 96°C in a sealed NMR tube.

The ^{1}H NMR spectrum of the solution was recorded at certain times (after transient cooling to 25°C), as exemplified in Fig. 1 for ribose at initial pD 4.5. In each spectrum, the pentose, 2-furaldehyde (7), and the formic acid ($^1HCO_2{}^2H$) with intact formyl proton were quantified. The results are shown in Figs. 2 and 3.

The isomeric composition of aldopentoses [11] and ketopentoses [12] in deuterium oxide solution at equilibrium has been determined previously, and the ^{1}H NMR spectra of their major isomeric forms have been analysed [13]. This was very helpful in the interpretation of our spectra. For all pentose signals, our chemical shifts (δ) were 0.02-0.03 ppm lower than those reported [13,14], probably owing to the buffer. In each spectrum except for that of ribulose, it was possible to select two or more regions, which were mutually well resolved and unaffected by hydrogen exchange with the solvent or by overlapping signals from the solvent (HDO) or degradation products. Each of these regions was integrated. Their assignments and positions are listed in Table 1. During each experiment, the ratios of the integrals remained constant within ±5%. The pentose concentration was therefore calculated from the average of the integrals.

In 7, H-4 (δ 7.91) and H-5 (δ 6.75) were integrated; both gave virtually the same results. The formyl proton (δ 8.33) was used in formic acid, and the methyl groups (δ 1.19) in the internal standard, pivalic acid.

As shown in Fig. 2, all three aldoses decomposed significantly, ribose being the most sensitive. Only traces of ketopentoses were observed in the spectra. This could be due to their high degradation rate under the same conditions, see Fig. 3. As shown by Figs. 2 and 3, increasing pD strongly accelerated the degradation of the aldoses but slightly retarded that of the ketoses. At pD 1.5, the formation of 7 was fast from the ketoses, slower from ribose, and even slower from the other aldoses. Increasing pD retarded the formation of 7, notably from the ketoses.

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[1] pD = observed pH meter reading + 0.4 [10]. pD also equalled pH of a similar solution of the undeuterated solutes in ordinary water.

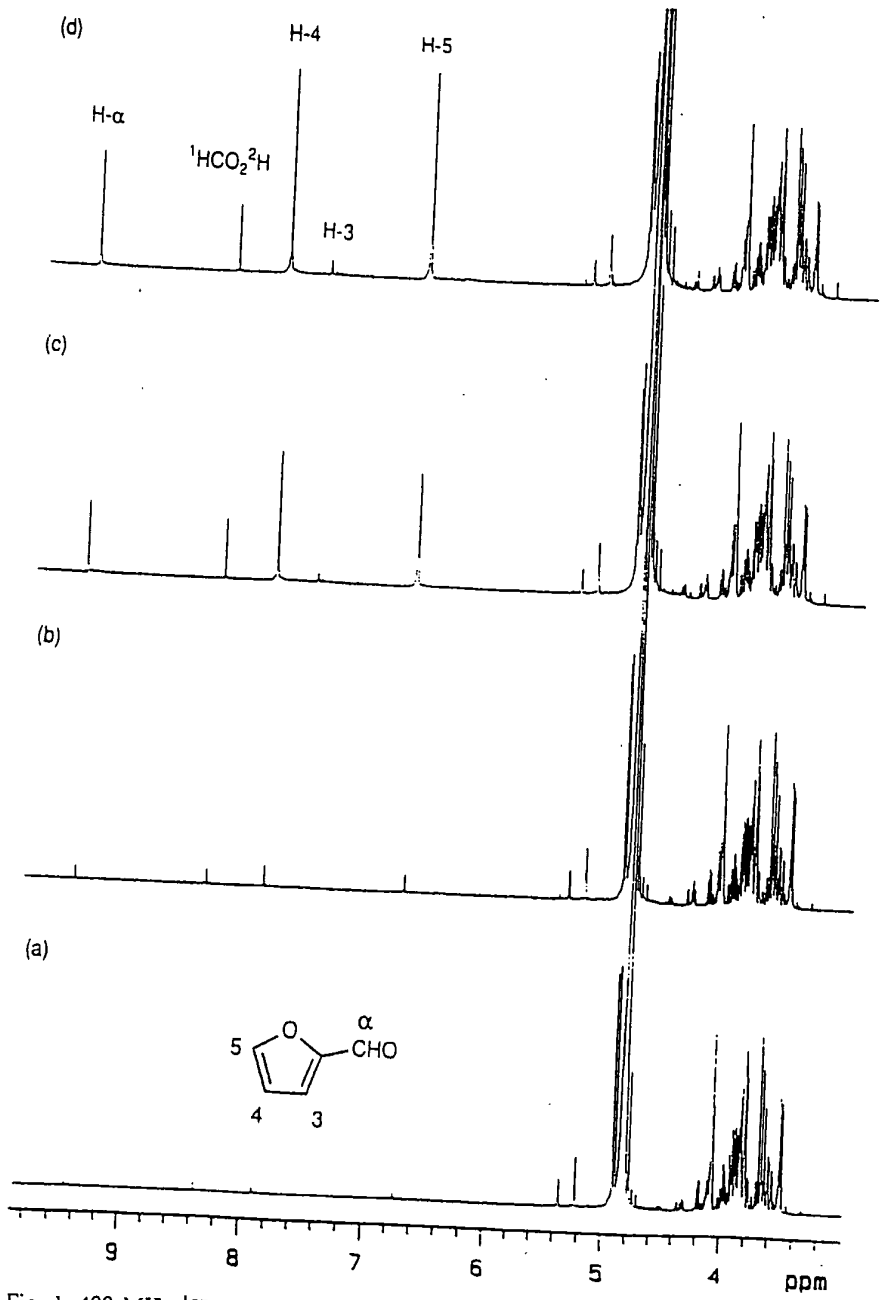


Fig. 1. 400 MHz ^1H NMR spectrum of D-ribose in 0.5 M deuterated sodium phosphate buffer (pD 4.5) at 25°C after heating at 96°C for (a) 2 h, (b) 8 h, (c) 48 h, and (d) 72 h.

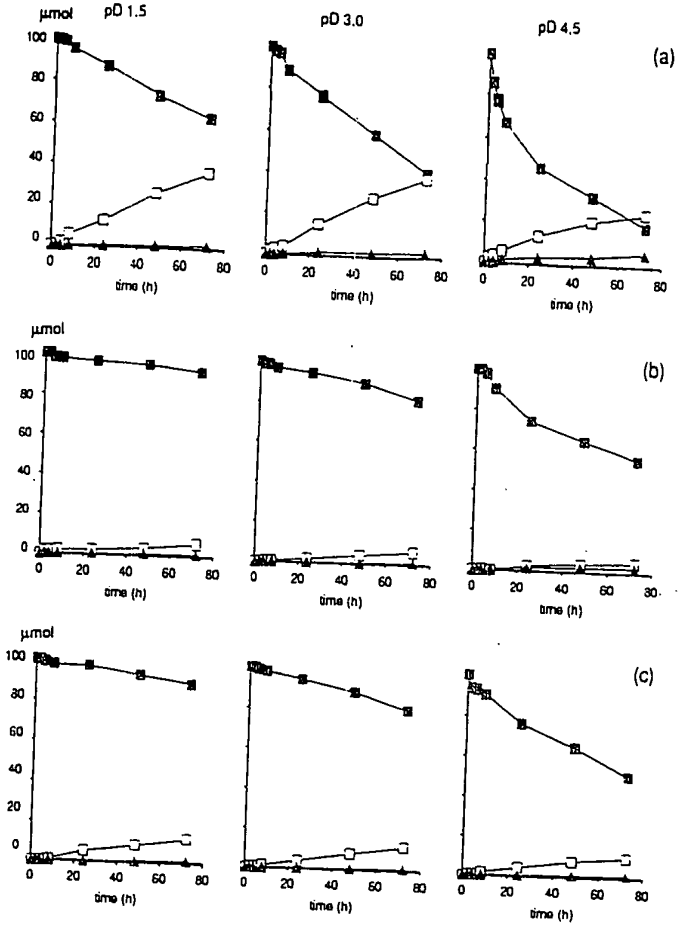


Fig. 2. Amounts of pentose (■), 2-furaldehyde (7, ❑), and intact formic acid ($^1HCO_2{}^2H$, ▲) after heating 100 µmol of (a) D-ribose, (b) D-arabinose, or (c) D-xylose in 0.5 M deuterated sodium phosphate buffer at 96°C.

No correction for any decomposition of 7 has been made in Figs. 2 and 3. When 7 was heated in phosphate buffers in the same way as the aldopentoses, <10% of 7 disappeared, see Fig. 4. In the pentose degradation experiments, additional 7 might have disappeared through reaction with other degradation products. However, the yields of 7, calculated on consumed pentose and shown in Fig. 5, indicated that very little 7 reacted further. Thus, nearly quantitative such yields of 7 were obtained from the aldopentoses after 72 h at pD 1.5, although Fig. 4 indicates maximum decomposition (9%) of 7 under these conditions. With increasing pD, these yields of 7 dropped quickly (Fig. 5), owing to the formation of other products [8]. These were indicated by strong colour of the solution and by a dark precipitate.

Table 1
Integrated regions in the ^1H NMR spectra of D-pentoses

D-Pentose	Proton/form	δ
Arabinose	H-2/α-*p*	3.46-3.49
Arabinose	H-1/α-*p*	4.48-4.51
Arabinose	H-1/β-*p*, H-1/α-*f*, H-1/β-*f*	5.19-5.45
Ribose [a]	H-2/β-*p*	3.49-3.54
Ribose [a]	H-2/α-*f*, H-3/α-*f*, H-3/β-*p*, H-4/α-*f*	4.05-4.15
Ribose [a]	H-1/β-*f*	5.23-5.25
Ribose [a]	H-1/α-*f*	5.36-5.38
Xylose	H-2/β-*p*, H-5b/β-*p*	3.16-3.36
Xylose	H-3/β-*p*	3.37-3.42
Xylose	H-1/β-*p*	4.53-4.56
Xylose	H-1/α-*p*	5.14-5.17
Ribulose	H-4/α-*f*, H-3/keto	4.24-4.39
Xylulose	H-3/β-*f*, H-3/α-*f*, H-4/keto	3.94-4.08
Xylulose	H-4/β-*f*	4.29-4.39

[a] See Fig. 1.

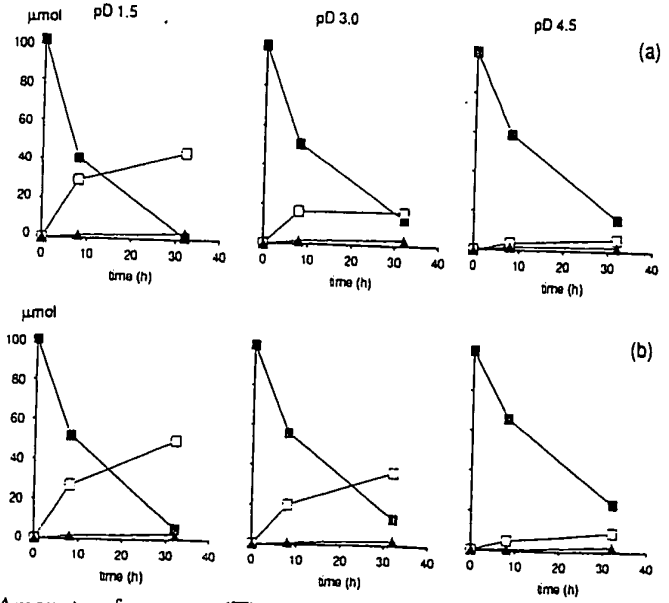


Fig. 3. Amounts of pentose (■), 2-furaldehyde (7, ❑), and intact formic acid ($^1HCO_2{}^2H$, ▲) after heating 100 µmol of (a) D-ribulose or (b) D-xylulose in 0.5 M deuterated sodium phosphate buffer at 96°C.

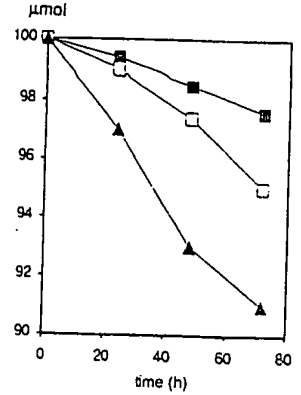


Fig. 4. Amounts of 2-furaldehyde (7) after heating 7 (100 µmol) in 0.5 M deuterated sodium phosphate buffer [pD 1.5 (▲), 3.0 (❑), or 4.5 (■)] at 96°C.

In the aldoses, virtually no hydrogen exchange with the solvent occurred in any of the experiments. In the ketoses, only H-1a and H-1b were exchanged: at pD 3.0 or 4.5, their signals had disappeared after 32 h.

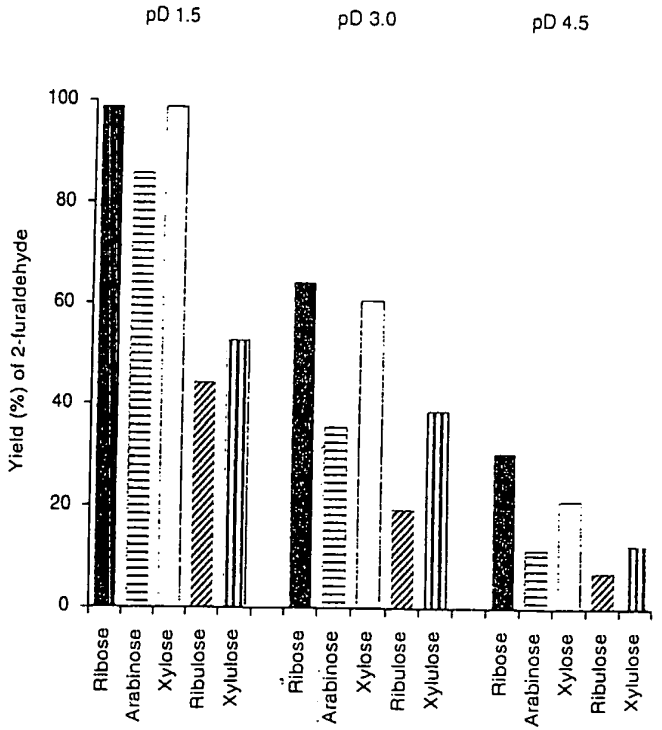


Fig. 5. Yields of 2-furaldehyde (7) calculated on consumed D-pentose at the end of the degradation experiments illustrated in Figs. 2 and 3.

Table 2

^2H content (p_α and p_3) of H-α and H-3 in 2-furaldehyde (7) formed from D-pentoses in 0.5 M deuterated sodium phosphate buffer at 96°C. The data were obtained by integration of the ^1H NMR spectra

D-Pentose	pD	p_α/atom %	p_3/atom %
Arabinose	1.5	25	100
Arabinose	3.0	39	100
Arabinose	4.5	61	100
Ribose	1.5	15	81
Ribose	3.0	42	87
Ribose	4.5	57	100
Xylose	1.5	15	98
Xylose	3.0	29	100
Xylose	4.5	50	100
Ribulose	1.5	9	79
Ribulose	3.0	39	97
Ribulose	4.5	83	100
Xylulose	1.5	8	86
Xylulose	3.0	35	96
Xylulose	4.5	74	100

As seen from the signals at δ 9.49 and 7.58 in Fig. 1, part of H-α and most of H-3 in 7 had been replaced by deuterium. The deuterium contents of H-α and H-3 were calculated from the relative decrease of these signals. The results are listed in Table 2. The higher the pD, the more deuterium was incorporated and at pD 4.5, the exchange of H-3 was virtually complete.

Formic acid was formed slowly in all the degradation experiments. Notably, no formic acid was formed from 7 under any of the conditions used. For further information on the reaction mechanism, some 1- and 5-^{13}C-substituted aldopentoses were degraded for 72 h in the same way as the ordinary aldoses. The ^1H and the proton-decoupled ^{13}C NMR spectra of the final reaction mixtures were recorded, and the formyl signals integrated.

The isotopic composition of carbon in the formic acid ($^1HCO_2{}^2H$) with intact formyl proton was determined by integration of the ^1H NMR signals (a $^{12}C^1H$ singlet and a $^{13}C^1H$ doublet). The results are listed in Table 3.

Table 3

^{13}C content (p) of carbon in intact formic acid ($^1HCO_2{}^2H$) formed from ^{13}C-substituted D-pentoses in 0.5 M deuterated sodium phosphate buffer at 96°C. The data were obtained by integration of the ^1H NMR signals (a $^{12}C^1H$ singlet and a $^{13}C^1H$ doublet) from the formyl group

D-Pentose	pD	p/atom %
(1-^{13}C)Ara	1.5	60
(5-^{13}C)Ara	1.5	50
(1-^{13}C)Ara	3.0	63
(5-^{13}C)Ara	3.0	37
(1-^{13}C)Ara	4.5	54
(5-^{13}C)Ara	4.5	52
(1-^{13}C)Rib	1.5	56
(5-^{13}C)Rib	1.5	51
(1-^{13}C)Rib	3.0	60
(5-^{13}C)Rib	3.0	48
(1-^{13}C)Rib	4.5	48
(5-^{13}C)Rib	4.5	56

From the integrals of the ^{13}C signals (a $^{13}C^1H$ singlet and a $^{13}C^2H$ triplet), the approximate deuterium content was estimated. This was 55 atom % at pD 4.5 and ≤35 atom % at pD 3.0, when starting from a (1-^{13}C)aldopentose. Unfortunately, the ^{13}C NMR spectra were too noisy for more precise determinations. At pD 1.5, no $^{13}C^2H$ signal from the formic acid was detected. This was also the case, when starting from a (5-^{13}C)aldopentose, even at pD 4.5. (No deuterium was incorporated into the formyl group when formic acid was heated for 72 h in deuterated phosphate buffer at pD 4.5.)

The highest yield of $^1HCO_2{}^2H$ (8.1 μmol from 100 μmol of consumed pentose) was obtained from ribose after 72 h at pD 4.5 (Figs. 2 and 3). The part (48%) of this acid derived from C-1 of the ribose was accompanied by a somewhat larger amount of $^2HCO_2{}^2H$. Hence, the highest yield of total formic acid from 100 μmol of consumed pentose was ~ 13 μmol.

3. Discussion

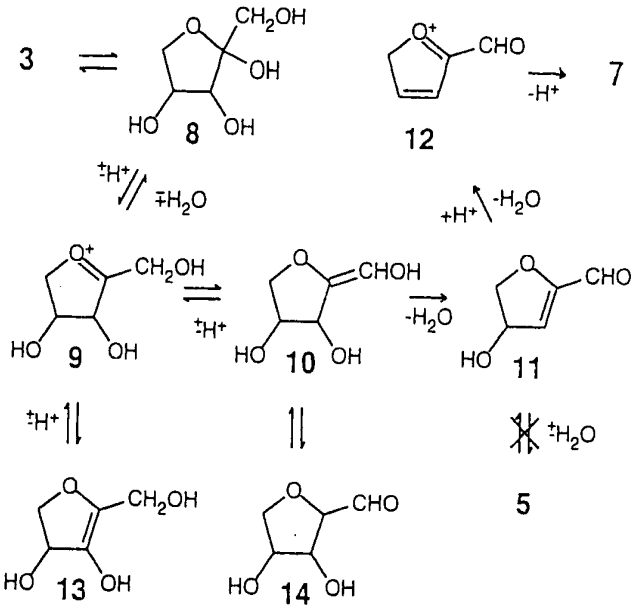
For each pentose except ribulose, the integrated regions (Table 1) included signals from more than one of the isomeric forms. Since the ratios of the integrals remained constant within ±5% during each experiment, mutarotation was much faster than degradation and remained close to equilibrium throughout the experiment.

The results shown in Fig. 2 indicate that the stability of aldopentoses in aqueous solution is highest at a surprisingly low pH value (near or below pH 1.5). Since increasing pD accelerated their degradation but retarded the formation of 2-furaldehyde (7), other products [8] must have formed at a strongly increasing rate. Among these products, only formic acid was formed in amounts sufficient to permit direct quantification by ^{1}H NMR spectroscopy.

As expected from Scheme 1, the relative degradation rate of each pentose seemed to be governed by the abundance of its acyclic form at equilibrium, which in turn was inversely related to the stability of its pyranose forms. Thus, the *all-cis*-configuration of α-D-ribopyranose leads to a *syn*-1,3-diaxial interaction in both chair conformers, resulting in a lower proportion of this isomer and a higher proportion of all other isomers, icluding the aldehydo isomer, compared to the other aldopentoses [11]. This may explain why ribose was degraded faster than arabinose and xylose. Since no pyranose forms of the ketopentoses exist, these contain an exceptionally high proportion of keto form [12] with prominent signals in the ^{1}H NMR spectrum. Accordingly, the ketopentoses were degraded much faster than even ribose.

The data in Table 2 are also compatible with the classical route to 7 (Scheme 1). Since most or all of H-3 was exchanged in 7, the enol 5 tautomerized faster than it was dehydrated, contrary to its behaviour in stronger acid [5]. In other words, the formation of 6 from 5 through β-elimination was slow enough to permit complete or nearly complete equilibration of 5 with 4. Similarly, the partial exchange of H-α in 7 implies that the 1,2-enediol 2 tautomerized to a ketose (3) faster than it was dehydrated to 5, although equilibrium between 2 and 3 was not attained. The gradual exchange of H-1a and H-1b in 3 confirms this interpretation. On the other hand, no hydrogen was exchanged in the aldoses (1). Hence, these were converted irreversibly to 1,2-enediol 2. Since H-3 was not exchanged in 3, any formation of 2,3-enediol from 3 was irreversible.

When "Amadori compounds" (1-amino-1-deoxyfructose derivatives) were degraded in deuterated 2 M acetic acid [15], partial exchange of H-α and H-3 in the resulting 5-(hydroxymethyl)-2-furaldehyde was observed and explained essentially as above.



Scheme 2. Alternative route from pentoses to 2-furaldehyde via ketopentofuranoses.

Although our results are well explained by Scheme 1, they do not rule out an alternative route via the ketofuranose 8 (which is more abundant than even the keto form 3 [12]) and the cyclic intermediates 9-12 (Scheme 2). An analogous route from fructose to 5-(hydroxymethyl)-2-furaldehyde has been proposed several times [4], most recently in 1990 [16]. Scheme 2 could apply to the aldopentoses as well, if these were converted into 8 via 1-3 or into 10 via the 2,5-anhydropentoses 14 through ring contraction of the pyranoses [7].

Cyclic intermediates have been proposed partly in order to explain why no hydrogen from the solvent is found at carbon in 7 or its 5-(hydroxymethyl) derivative. However, the conclusions are based on experiments [5-7,16] performed in strong acid and/or at very high temperature. Under the milder conditions used in the present study, 10 might be dehydrated slowly enough to permit its reversible conversion into 13 via 9, explaining the data in Table 2.

If this interpretation were correct, 10 should also have time to tautomerize to 14. However, 14 was not observed in the ^{1}H NMR spectra. Similarly, the analogue of 14, 2,5-anhydromannose, was not observed in the degradation of fructose and was less reactive than fructose [4]. Moreover, neither 4 nor 5 is an intermediate in Scheme 2, although 4 is no doubt involved in Maillard reactions [2] and in carbohydrate degradation under basic conditions [4]. For these reasons, we prefer to interpret our results according to Scheme 1. The route from pyranoses via 14 to 7 [7] does also not explain why ribose is degraded faster than arabinose and xylose.

The results in Table 3 indicate that the formic acid was derived exclusively from the terminal pentose carbons, C-1 being somewhat more important than C-5. The apparently exceptional data obtained at pD 4.5 are explained by the large proportion of fully deuterated formic acid ($^2HCO_2{}^2H$) at pD 4.5, for this acid was derived mainly from C-1 of the pentose [2]. Despite these results, we are still far from knowing how the formic acid is formed.

Isotope effects have been disregarded in the above discussion. The $^{13}C/^{12}C$ effects were probably not much larger than those introduced by errors in the integrals of the NMR signals. The $^2H/^1H$ effects were certainly larger but do not affect our qualitative conclusions.

4. Experimental

Materials. - All reagents and solvents were commercial samples of analytical grade. The ^{13}C-substituted pentoses were obtained from Omicron Biochemicals, Inc. Their isotopic purity was 99 atom % and that of the deuterium oxide 99.9 atom %. Orthophosphoric acid and its salts were deuterated by repeated rotary evaporation of their solutions in deuterium oxide. The buffers were prepared by adding 0.5 M D_3PO_4 or Na_2DPO_4 to 0.5 M NaD_2PO_4, until the pH meter showed the desired value (pD - 0.4) [10]. Pivalic acid (1.00 mg/mL) and sodium 3-(trimethylsilyl)propionate-d_4 (TSP, 1.00 mg/mL) were added to each buffer. This lowered the pH meter reading by 0.04.

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[2] This is exemplified by the results for ^{13}C-substituted ribose at pD 4.5 (last two lines in Table 3). For simplicity, it is assumed that the formic acid was derived from C-1 or C-5, and that all $^2HCO_2{}^2H$ was derived from C-1, as indicated by the results. Moreover, ~ 55 mole % of the formic acid derived from C-1 was $^2HCO_2{}^2H$. Hence, 45 mole % of it was $^1HCO_2{}^2H$. Out of 100 mol of $^1HCO_2{}^2H$ formed from (1-^{13}C)ribose, 48 mol was derived from C-1 and, hence, 52 mol from C-5. The total amount of formic acid derived from C-1 was therefore 48/0.45 = 107 mol. Hence, 100·107/(107 + 52) = 67 mole % of the formic acid was derived from C-1 and 33 mole % from C-5. Similarly, out of 100 mol of $^1HCO_2{}^2H$ formed from (5-^{13}C)ribose, 56 mol was derived from C-5 and, hence, 44 mol from C-1. The total amount of formic acid derived from C-1 was therefore 44/0.45 = 98 mol. Hence, 100·98/(98 + 56) = 64 mole % of the formic acid was derived from C-1 and 36 mole % from C-5. Without more accurate results on the deuterium content of the formic acid, we made no similar calculations from the other data in Table 3.

^1H NMR spectra. - These were recorded at 400 MHz and 25°C on a Varian VXR-400 instrument and referenced to internal TSP. A 45° pulse and a 6.3 s relaxation delay were applied to allow complete relaxation. The FID data were transferred to the program NMR1 (from New Methods Research, Inc.) and then processed. The line-broadening factor was 0.3 Hz. The integrals of relevant signals were measured after performing the baseline flatting routine both automatically and manually. The integrals were calculated automatically, after the spectral lines had been identified. For each line, the integral was calculated over 5 line widths. The integral phase correction was carried out manually.

^{13}C NMR spectra. - These were recorded using inverse gated decoupling, a relaxation delay of 10 s and a pulse width of 45°. The conditions were the same as in the ^1H NMR experiments.

Degradation experiments. - In each experiment, a pentose (15.0 mg, 100 μmol) or 7 (9.6 mg, 100 μmol) was dissolved in the appropriate buffer (0.60 mL) and transferred to a 5 mm NMR tube. The tube was sealed, the ^1H NMR spectrum recorded, and the tube heated in a water bath at 96°C. At certain times (t), the tube was cooled quickly. After 1-2 h at 25°C, the ^1H NMR spectrum was recorded again, and the heating continued immediately. When an aldose was degraded, t was 2, 4, 8, 24, 48, and 72 h; when a ketose was degraded, t was 8 and 32 h; and when 7 was degraded, t was 24, 48, and 72 h. The heating times t are indicated in Figs. 1-4 and do not include the waiting times at 25°C. The data in Table 2 were obtained from the final spectra (after 32 or 72 h) and have been corrected for the ^1H content (0.7 atom %) of the medium (mainly due to pentose-O^1H). When the (^{13}C)aldopentoses were degraded, ^1H and ^{13}C NMR spectra were recorded only after 72 h. The data in Table 3 have been corrected for the ^{12}C content (1 atom %) of the ^{13}C-substituted carbons.

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II

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On the formation of 2,3-dihydroxyacetophenone from pentoses or hexuronic acids

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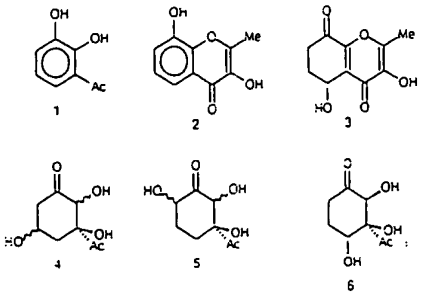
ABSTRACT

The structure of a previously isolated intermediate in the title reaction has been revised to 3-acetyl-2,3,4-trihydroxycyclohexanone by high-field ^{1}H NMR spectroscopy. The three hydroxyl groups are mutually *cis*-related.

INTRODUCTION

About 20 years ago, catechol and a number of its derivatives were obtained by degradation of pentoses or hexuronic acids in slightly acidic, aqueous solution[1,2]. Among these derivatives, 2,3-dihydroxyacetophenone (1) and 3,8-dihydroxy-2-methylchromone (2) were the most abundant. The chromone 2 was the only phenolic compound previously[3] obtained in a similar way.

A clue to the mechanism of formation of 1 and 2 was found by the trapping of alicyclic intermediates. Thus, formula 3 † was assigned to a precursor of 2[2]. For a precursor of 1, formulas 4[1] and 5[2] were proposed, without indication of the stereochemistry. In the present paper, this precursor of 1 is shown to have the structure and stereochemistry indicated by formula 6.



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† All chiral compounds were obtained as racemic mixtures, but only one enantiomer is shown.

RESULTS AND DISCUSSION

Succinic acid had previously[2] been obtained from the precursor of 1 on borohydride reduction, followed by oxidation with lead(IV) acetate and with oxygen on platinum. This result ruled out formula 4, but was equally consistent with 5 and 6.

The ^{1}H NMR spectra of the originally[2] isolated precursor and its diacetate were complicated by extensive overlap, even at 400 MHz (Table I). However, in the spectrum (Fig. 1) of the diacetate, dissolved in a mixture of chloroform and acetonitrile, the signals were separated well enough to permit a clear decision between formulas 5 and 6 in favour of the latter. The symbol 6a is therefore used for the diacetate.

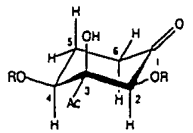
The methylene protons were readily assigned owing to the strong coupling between axial and between geminal protons. The fairly high values (δ 2.46 and 2.60, $|^2J|$ 15.0 Hz) observed for one methylene group indicated that this was adjacent to the carbonyl group. More important, when the axial proton of this methylene group was saturated, NOEs of both methine signals were observed. Hence, the methine protons were also axial. Moreover, a substantial coupling ($|^4J| \sim 1.0$ Hz) between the irradiated proton and one methine proton was observed. Such long-range coupling between axial protons implies that these are

TABLE I

^{1}H NMR data for *r*-3-acetyl-*t*-2,3,*t*-4-trihydroxycyclohexanone (6) in CD_3OD at 25°C and for its diacetate (6a) in 5:1 $CDCl_3$–CD_3CN at 60°C, see Fig. 1

H	δ		H, H	$\|J\|$ (Hz)	
	6a	6		6a	6
H-2	5.58	4.64	2, 6*a*	0.8 [a]	1.2
H-4	5.61	4.48	4, 5*a*	11.7	8.8
H-5*a*	2.17	2.04	4, 5*e*	5.1	7.4
H-5*e*	2.23	2.04	5*a*, 5*e*	12.7	
H-6*a*	2.60	2.57	5*a*, 6*a*	14.3	11.5
H-6*e*	2.46	2.31	5*a*, 6*e*	4.8	
Ac-3	2.27	2.35	5*e*, 6*a*	6.3	
AcO	2.00		5*e*, 6*e*	2.1	2.3
AcO	2.09		6*a*, 6*e*	15.0	14.5
HO	4.1				

[a] 1.2 Hz in pure $CDCl_3$.



6 R = H
6a R = Ac

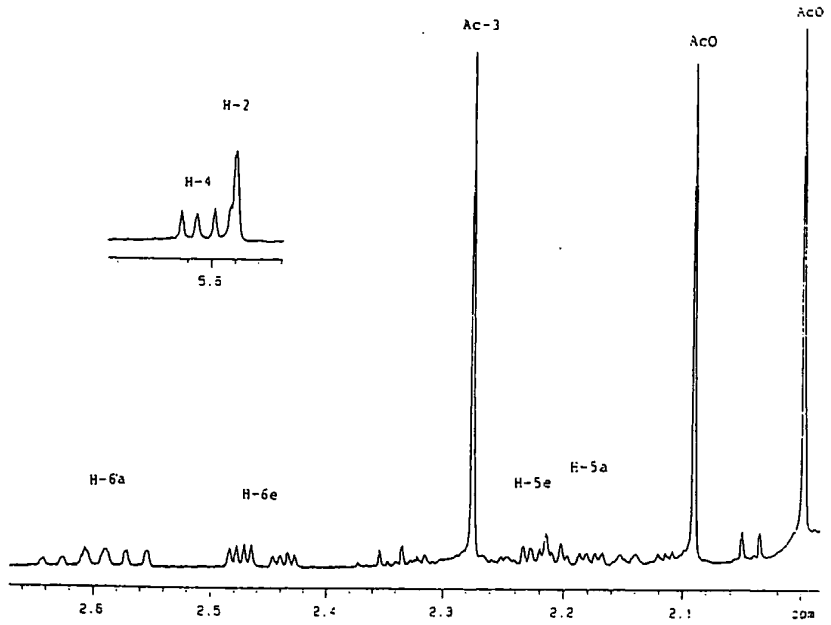
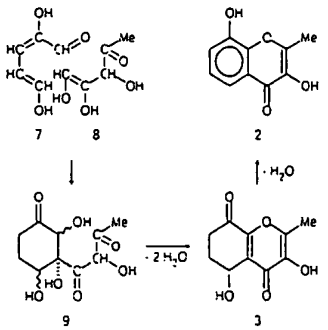


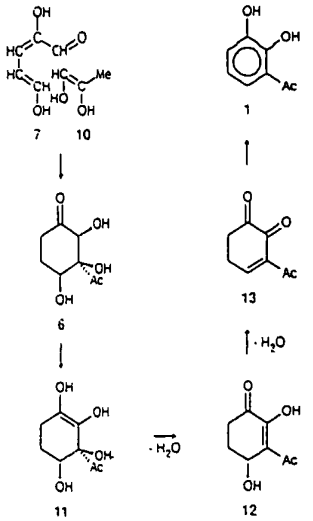
Fig. 1. 400-MHz ^{1}H NMR spectrum of compound 6a in 5:1 $CDCl_3$–CD_3CN at 60°C.

linked through the carbonyl group[4], which is consistent with 6a but not with a diacetate of 5. Finally, saturation of the axial proton of the other methylene group caused an NOE of the signal from the unacetylated hydroxyl group, which was therefore axial. This was confirmed by weak NOEs of the methine signals on saturation of the *C*-acetyl protons. The various NOEs were seen most clearly in the NOE difference spectra.

A route to 2 via 3 has been proposed previously[5]. It is shown in Scheme 1 after slight modification. The key step, joining two C_5 species, is a Michael addition of



Scheme 1. Key and subsequent steps in the formation of chromone 2 via 3.



Scheme 2. Key and subsequent steps in the formation of 2,3-dihydroxyacetophenone (1) via 6.

an enediol (8) to an unsaturated aldehyde (7), followed by cyclization through aldol condensation. The subsequent steps are simple tautomerizations and β-eliminations. The route in Scheme 2 was later[6] supported by isotopic tracer techniques. Aldehyde 7 has also been proposed as a precursor of the so-called reductic acid[7].

An analogous route to 1 via 6 is shown in Scheme 2. Here, the key step joins 7 and the enediol 8 of acetol, which is a common sugar dehydration product[8]. Again, the subsequent steps are simple tautomerizations and β-eliminations. Such a simple route to 1 via 4 or 5 is not easy to imagine. In order to confirm the route in Scheme 2, we have tried to prepare 1 from acetol and some compound similar to 7 but, so far, only products related to reductic acid have been obtained[9]. Nevertheless, 7 may be a more general precursor of other phenolic products in the title reaction.

EXPERIMENTAL

The samples of **6** and **6a** were prepared in 1971[2] (and were then regarded as 5 and its diacetate). The ^{1}H NMR spectra were recorded with a Varian VXR-400 instrument and referenced to the solvent $CDCl_3$ ($CHCl_3$, δ 7.26) or CD_3OD (CHD_2OD, δ 3.31). The chemical shifts (δ) and coupling constants (J) are listed in Table I. The data for the ring protons of **6a** were obtained by spin simulation, using the standard software from Varian, VnmrS 4.1A. The spectrum of **6a**, dissolved in 5:1 $CDCl_3$–CD_3CN to minimize overlap, is shown in Fig. 1. NOE difference spectra were recorded using the same solvent mixture degassed with Ar, and a saturation time of 20 s.

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III

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On the formation of reductic acid from pentoses or hexuronic acids

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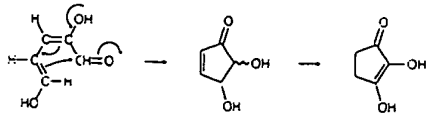
ABSTRACT

Careful hydrolysis of (±)-*cis*- or (±)-*trans*-tetrahydro-2,5-dimethoxy-2-furaldehyde dimethyl acetal proceeded via 5,5-dimethoxy-4-oxopentanal to give (±)-*trans*-4-hydroxy-5-methoxy-2-cyclopentenone and (±)-*trans*-4,5-dihydroxy-2-cyclopentenone. The latter product did not isomerize to 2,5-dihydroxy-2-cyclopentenone (reductic acid) on prolonged reaction.

INTRODUCTION

Although 2,5-dihydroxy-2,4-pentadienal has never been isolated, some of its geometrical isomers have been proposed as key intermediates in the degradation of pentoses or hexuronic acids. Thus, one isomer (1) is believed[1] to cyclize by aldol condensation to the cyclopentenone 2 [*], which then tautomerizes to the so-called reductic acid (3), see Scheme 1. The stereochemistry of 2 is not known.

Isomer 1 has also been proposed as a precursor of certain phenolic degradation products[2–4]. In order to confirm this, we needed a compound related to 1 but stable enough to permit isolation. For this purpose, tetrahydro-2,5-dimethoxy-2-furaldehyde dimethyl acetal (6 in Scheme 2) was chosen, because its ethyl analogue had been converted previously into a phenolic compound (3,8-dihydroxy-2-methylchromone)[3]. The present paper deals with the preparation and hydrolysis of 6. The hydrolysis invariably produced the *trans* isomer (2t) of 2, but no reductic acid (3).



1 2 3

Scheme 1. Proposed formation mechanism of reductic acid.

* Corresponding author.

[*] All chiral compounds were obtained as racemic mixtures, but only one enantiomer is shown.

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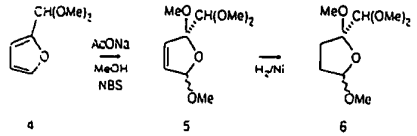
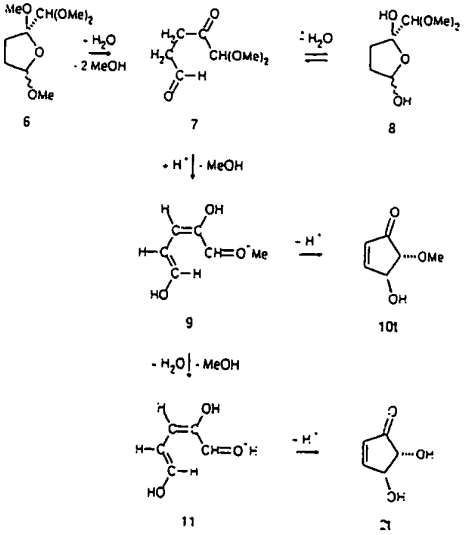


Scheme 2. Preparation of compound 6 (diastereomeric mixture).

RESULTS AND DISCUSSION

Compound 6 has been prepared from 2-furaldehyde dimethyl acetal (4) via the 2,5-dihydro derivative[5] (5), see Scheme 2. After some improvements, we preferred a procedure described for the ethyl analogue[2]. Compound 5 was fractionated by chromatography into the known[6] *cis* and *trans* isomers (5h6c and 5t), which were hydrogenated to the corresponding isomers (6c and 6t) of 6. Since the isomers 6c and 6t behaved in the same way as their mixture (6) on hydrolysis, only the hydrolysis of 6 will be described.

Careful hydrolysis converted 6 into the new 5,5-dimethoxy-4-oxopentanal (7) and its diastereomeric hydrates (8), see Scheme 3. The ^1H and ^{13}C NMR spectra showed 7 in chloroform solution but 8 in aqueous solution, as evidenced by the presence or absence of the aldehyde proton (H-1) and the two carbonyl carbons. The spin–spin coupling between H-1 and the adjacent methylene protons (H-2) was too weak to cause splitting of the H-1 signal but was revealed by sharpening of the H-2 signal on irradiation at the frequency of H-1.



Scheme 3. Hydrolysis of compound 6.

On prolonged reaction, 7 gradually cyclized to 2t and its monomethyl ether 10t, but no reductic acid (3) was detected. The cyclizations of 7 are apparently aldol condensations via cationic analogues (9 and 11) of 1. Owing to rapid tautomerization, the ^1H NMR spectrum of 3 in D_2O consists of a single sharp line[7]. The ^1H NMR spectrum of 2t was much more informative and strongly resembled that of its *cis* isomer[8]. However, the two spectra showed different coupling constants (J), especially for coupling between the saturated methine protons (H-4 and H-5). Thus, $J_{4,5}$ 2.7 Hz was observed for 2t and $J_{4,5}$ 5.3 Hz for the *cis* isomer. The *cis* configuration of the latter isomer followed from its synthesis via the 4,5-di-*O*-isopropylidene derivative[8]. Hence, 2t is the *trans* isomer. Since the four coupling constants observed for 10t agreed, within ±0.1 Hz, with those observed for 2t, 10t is also the *trans* isomer. The ^{13}C NMR spectra of 2t and 10t, together with the ^1H NMR spectra of their acetates, further supported their formulas.

EXPERIMENTAL

General methods.—Solvent mixtures are defined by volume ratios (v/v). The light petroleum used boiled at 60–70°C. Concentration was conducted under diminished pressure below 40°C. The progress of all reactions was monitored by TLC using Silica Gel HF_{254} (Merck) microplates and the following standard spray reagents[9]: *A*, *p*-anisidine · HCl; *B*, resorcinol–aq HCl; and *C*, aq $FeCl_3$. Column chromatography was performed on silica gel (230–400 mesh, Merck). GLC was carried out at 100°C on a Packard 427 instrument fitted with a flame-ionization detector and a DB1 column (30 m × 0.22 mm; linear flow rate, 0.3 m/s). GLC–EIMS was performed at 70 eV. The low-resolution spectra were recorded with a Finnigan 4021 instrument. The high-resolution spectra were recorded with a magnetic sector instrument[10] at 100 μA and 200°C with perfluorotributylamine as a reference. IR spectra were scanned with a Perkin–Elmer 1760X FTIR spectrometer; no solvent was used. NMR spectra were recorded with a Varian VXR-400 instrument at 25°C. The samples were dissolved in $CDCl_3$ or D_2O. ^1H NMR spectra were recorded at 400 MHz and referenced to the solvent ($CHCl_3$, δ 7.26) or (in D_2O) to internal $Me_3SiCH_2CH_2CO_2Na$ (Me, δ 0.00). ^{13}C NMR spectra were recorded at 101 MHz and referenced to the solvent ($CDCl_3$, δ 77.1) or (in D_2O) to internal 1,4-dioxane (δ 67.4). For complete assignment of all signals in the di- and tetra-hydrofuran derivatives 5, 6, and 8, H–H COSY, C–H COSY, and long-range C–H COSY experiments were performed, using the standard software from Varian.

Reductic acid (3).—This was prepared according to Theander[11].

2-Furaldehyde dimethyl acetal (4).—This was prepared from the aldehyde according to a general procedure[12]; bp 55–58°C at 2.0 kPa; lit.[13] 57–58°C at 1.9 kPa.

2,5-Dihydro-2,5-dimethoxy-2-furaldehyde dimethyl acetal (5).—2-Furaldehyde dimethyl acetal (4; 7.1 g, 50 mmol) and NaOAc (10 g) were dissolved in MeOH (15

mL) and Et_2O (15 mL). NBS (*N*-bromosuccinimide, 8.9 g, 50 mmol) was added in small portions to the stirred solution (which was cooled in an ice bath) kept below 5°C. After 30 min, the mixture was concentrated, aq KOH (50 mL, 7 g, 0.12 mol) added, and the mixture extracted with CH_2Cl_2 (4 × 50 mL). The extract was dried (K_2CO_3) overnight and filtered, the solvent evaporated, and the residue distilled to give a colourless liquid (5.49 g, 58% yield); bp 100–105°C at 2.0 kPa. This liquid was fractionated on a silica gel column (80 × 5 cm) with 5:1 light petroleum –EtOAc. Two chromatographically pure compounds (**5c** and **5t**) were obtained, giving a violet colour with spray *B*, and having the same mass spectrum, *m/z* (*rel* int.): 173 (M – MeO˙, 2) 129 (10), 101 (10), 75 (100). Their ^{1}H NMR spectra were in accordance with those reported[6].

The *cis* isomer **5c** (yield 14%) had R_f 0.24; GLC retention time, 9.0 min.

The *trans* isomer **5t** (yield 13%) had R_f 0.16; GLC retention time, 9.3 min.

Tetrahydro-2,5-dimethoxy-2-furaldehyde dimethyl acetal (**6**).—A mixture (27.35 g, 0.14 mol) of **5c** and **5t** in dry MeOH (400 mL) was shaken with Raney nickel under H_2 gas overnight under ambient conditions. The solvent was evaporated and a colourless liquid isolated by distillation (25 g, 91% yield); bp 100–110°C at 2.0 kPa. This liquid was fractionated on a silica gel column with 5:1 light petroleum–EtOAc. Two chromatographically pure compounds (**6c** and **6t**) were obtained, giving a greyish-red colour with spray *B* and having the same mass spectrum, *m/z* (*rel* int.): 206 (M, 0.5), 175 (3), 144 (5), 131 (42), 115 (12), 99 (18), 75 (100), 71 (88). Their configurations were established by hydrogenation of **5c** and **5t** separately on a smaller scale.

The *cis* isomer **6c** had R_f 0.35; GLC retention time, 9.3 min; ^{1}H NMR ($CDCl_3$): δ 1.88–2.18 (m, 4 H, H-3,4), 3.36 (s, 3 H, OMe-2), 3.43 (s, 3 H, OMe-5), 3.49 and 3.52 (2 s, each 3 H, OMe-α), 4.22 (s, 1 H, H-α), 5.05 (dd, 1 H, *J* 1.9 and 4.9 Hz, H-5); ^{13}C NMR ($CDCl_3$): δ 27.0 (C-3), 32.4 (C-4), 49.6 (OMe-2), 55.4 (OMe-5), 56.4 and 58.1 (OMe-α), 106.3 (C-α), 106.5 (C-5), 111.2 (C-2).

The *trans* isomer **6t** had R_f 0.19; GLC retention time, 8.9 min; ^{1}H NMR ($CDCl_3$): δ 1.82–2.28 (m, 4 H, H-3,4), 3.30 (s, 3 H, OMe-2), 3.39 (s, 3 H, OMe-5), 3.52 and 3.53 (2 s, each 3 H, OMe-α), 4.27 (s, 1 H, H-α), 5.17 (dd, 1 H, *J* 1.0 and 5.3 Hz, H-5). ^{13}C NMR ($CDCl_3$): δ 26.0 (C-3), 31.6 (C-4), 49.7 (OMe-2), 53.3 (OMe-5), 54.8 and 55.2 (OMe-α), 106.25 (C-α), 106.3 (C-5), 110.0 (C-2).

5,5-Dimethoxy-4-oxopentanal (**7**).—A mixture (5 g, 24.27 mmol) of **6c** and **6t** was heated with water (50 mL) in a water bath at 96°C for 3 h, cooled, and extracted with $CHCl_3$. The extract was evaporated. The syrupy residue was fractionated on a column of silica gel with 10:1 CH_2Cl_2–Me_2CO to give **7** in 38% yield; R_f 0.16 (10:1 CH_2Cl_2–Me_2CO). It gave a yellow colour with spray *A* and a red colour with spray *B*; GLC retention time, 5.3 min; ν_{max} 1730 (C=O); ^{1}H NMR ($CDCl_3$): δ 2.78 (t, 2 H, H-2), 2.88 (t, 2 H, H-3), 3.42 (s, 6 H, Me), 4.54 (s, 1 H, H-5), 9.8 (s, 1 H, H-1); ^{13}C NMR ($CDCl_3$): δ 29.9 (C-2), 36.9 (C-3), 54.8 (Me), 103.7 (C-5), 200.2 (C-4), 203.9 (C-1); MS, *m/z* (*rel* int.): 159 (M – H˙, 0.1), 129 (M – MeO˙, 2), 97 (2), 85 (3), 75 (100); Calcd for $C_7H_{11}O_4$ (M – H˙): 159.066. Found: 159.061.

Tetrahydro-2,5-dihydroxy-2-furaldehyde dimethyl acetal (**8**).—Compound **7** was dissolved in water.

Major isomer. ^{1}H NMR (D_2O): δ 1.79–2.30 (m, 4 H, H-3,4), 3.558 and 3.561 (2 s, each 3 H, Me), 4.34 (s, 1 H, H-α), 5.60 (m, 1 H, H-5); ^{13}C NMR (D_2O): δ 30.9 (C-3), 31.8 (C-4), 58.3 and 58.5 (Me), 99.6 (C-5), 106.9 (C-2), 107.9 (C-α).

Minor isomer. ^{1}H NMR (D_2O): δ 1.94–2.25 (m, 4 H, H-3,4), 3.54 and 3.55 (2 s, each 3 H, Me), 4.30 (s, 1 H, H-α), 5.52 (m, 1 H, H-5); ^{13}C NMR (D_2O): δ 31.8 (C-3), 32.8 (C-4), 58.3 and 58.5 (Me), 100.4 (C-5), 107.0 (C-2), 107.5 (C-α).

Preparation of compounds **2t** *and* **10t**.—Compound **7** (450 mg, 2.8 mmol) was heated at 80°C in a water bath with Me_2CO (25 mL) and 2% H_2SO_4 (500 μL) for 6 h. The Me_2CO was evaporated. The residue was neutralized with aq Na_2CO_3 and extracted with $CHCl_3$. The extract was evaporated. The resulting syrup was fractionated on a column of silica gel (20 × 5 cm) and three main fractions were collected by elution with 3:2 CH_2Cl_2–Me_2CO. The first fraction gave **2t** in 12% yield; R_f 0.46 (3:2 CH_2Cl_2–Me_2CO), brown colour with spray *A* and red with *B*; GLC retention time, 3.6 min; ν_{max} 1730 (C=O), 1100 (C–OH), and 3400 cm^{-1} (OH); ^{1}H NMR ($CDCl_3$): δ 3.2 and 3.5 (2 bs, each 1 H, OH), 4.21 (d, 1 H, *J* 2.7 Hz, H-5), 4.82 (m, 1 H, *J* 1.4, 1.9, and 2.7 Hz, H-4), 6.28 (dd, 1 H, *J* 1.4 and 6.1 Hz, H-2), 7.47 (dd, 1 H, *J* 1.9 and 6.1 H, H-3); ^{13}C NMR (D_2O): δ 78.7 (C-4), 83.1 (C-5), 134.6 (C-3), 164.9 (C-2), 209.7 (C-1); MS, *m/z* (*rel* int.): 114 (2, M), 113 (3, M – H˙), 96 (80), 68 (100); Calcd for $C_5H_5O_3$ (M – H˙): 113.024. Found: 113.019.

Compound **2t** (10 mg) was acetylated with dry pyridine (1 mL) and Ac_2O (1 mL) overnight at room temperature. The solution was poured into ice–water and the resulting mixture extracted with $CHCl_3$. The extract was washed with 0.1 M HCl (2 × 25 mL), 0.1 M $NaHCO_3$ (2 × 5 mL), and water (2 × 2 mL), dried (Na_2SO_4), filtered, and evaporated. The diacetate was obtained in 90% yield; GLC retention time, 7.8 min; R_f 0.28 (3:1 light petroleum –EtOAc), red colour with spray *B*; ^{1}H NMR ($CDCl_3$): δ 2.10 and 2.17 (2 s, each 3 H, Ac), 5.18 (d, 1 H, *J* 3.0 Hz, H-5), 5.90 (m, 1 H, *J* 1.4, 2.2, and 3.0 Hz, H-4), 6.41 (dd, 1 H, *J* 1.4 and 6.1 Hz, H-2), 7.45 (dd, 1 H, *J* 2.2 and 6.1 Hz, H-3); MS, *m/z* (*rel* int.): 198 (M, 0.1), 156 (2), 138 (M – AcOH, 2), 96 (25), 68 (22), 43 (100); Calcd for $C_9H_{10}O_5$ (M): 198.053. Found: 198.048.

The second fraction above gave **10t** in 10% yield; R_f 0.76 (3:2 CH_2Cl_2–Me_2CO), no colour with spray *A*, red colour with spray *B*; GLC retention time, 4.3 min; ^{1}H NMR ($CDCl_3$): δ 3.4 (bs, 1 H, OH), 3.65 (s, 3 H, Me), 3.85 (d, 1 H, *J* 2.7 Hz, H-5), 4.82 (m, 1 H, *J* 1.3, 2.0, and 2.7 Hz, H-4), 6.20 (dd, 1 H, *J* 1.3 and 6.2 Hz, H-2), 7.40 (dd, 1 H, *J* 2.0 and 6.2 Hz, H-3); ^{13}C NMR ($CDCl_3$): δ 57.5 (Me), 75.6 (C-4), 88.4 (C-5), 133.2 (C-3), 159.0 (C-2), 199.0 (C-1); MS, *m/z* (*rel* int.): 128 (M, 54), 127 (13), 111 (7), 100 (39), 85 (57), 71 (75), 57 (100), 43 (71); Calcd for $C_6H_8O_3$ (M): 128.047. Found: 128.046.

Compound **10t** was acetylated as described for **2t**. The yield of the acetate was 90%; R_f 0.35 (3:1 light petroleum–EtOAc), red colour with spray *B*; GLC retention time, 6.5 min; ^{1}H NMR ($CDCl_3$): δ 2.10 (s, 3 H, Ac), 3.65 (s, 3 H, OMe),

3.96 (d, 1 H, *J* 2.7 Hz, H-5), 5.76 (m, 1 H, *J* 1.4, 2.1, and 2.7 Hz, H-4), 6.30 (dd, 1 H, *J* 1.4 and 6.2 Hz, H-2), 7.40 (dd, 1 H, *J* 2.1 and 6.2 Hz, H-3); MS, *m/z* (*rel* int.): 128 (M – CH_2CO, 19), 111 (M – AcO, 18), 110 (M – AcOH, 13), 96 (34), 68 (28), 55 (15), 43 (100); Calcd for $C_8H_{10}O_4$ (M): 170.058. Found: 170.058.

The third fraction above mainly contained unreacted **7**, which was recovered in 7% yield.

Hydrolysis and isomerization experiments.—(*a*) Compound **6** was heated in 0.1 M DCl (in D_2O) at 90°C in a water bath. The ^1H NMR spectrum was recorded after 1, 2, and 4 h, and showed increasing amounts of **7** and **8** at the expense of **6**. The last spectrum also showed traces of **2t**. No reductic acid (**3**) was detected on comparison with an authentic sample.

(*b*) Compound **7** was treated as for **6** in (a). Compounds **2t** and **10t** formed gradually at the expense of **7**. Again, no **3** was detected.

(*c*) Compound **2t** (100 mg) was heated at 50, 80, or 90°C in a water bath with 1 mL of 0.1 M H_2SO_4, glacial AcOH, 3.5 M HCl, or 0.5 M $NaHCO_3$. Each mixture was analyzed by TLC (3:2 CH_2Cl_2–Me_2CO) after 0.5, 1, 2, 3, and 4 h, using sprays *A*, *B*, and *C*. No **3** was detected, but the amount of **2t** decreased with time.

(*d*) Reductic acid (**3**) was treated as in (*c*). Its amount decreased with time, but no **2t** was formed.

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IV

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α-1,4-Glucan lyase, a new class of starch/glycogen degrading enzyme. III. Substrate specificity, mode of action, and cleavage mechanism

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Abstract

The α-1,4-glucan lyase (EC 4.2.2.-), purified from the red alga *Gracilariopsis lemaneiformis*, is a single polypeptide with a molecular mass of 116654 Da as determined by matrix-assisted laser-desorption mass spectrometry. It degraded maltose, maltosaccharides, amylose, amylopectin and glycogen, forming 1,5-anhydro-D-fructose from the non-reducing end groups. The substrate specificity, mode of action, and cleavage mechanism of the enzyme were studied by using various naturally occurring and synthesized substrates. This enzyme was highly specific for the α-1,4-D-glucosidic bond. When a linear α-1,4-glucan was used as substrate, the enzyme split the substrate from the non-reducing end and released 1,5-anhydro-D-fructose successively until only one glucose unit was left. When a branched pentasaccharide of 6^2-α-maltosylmaltotriose, obtained from glycogen by α-amylase limitation, was used as substrate, the glucose group in the 4-position of the 4,6-branched residue was not cleaved off. Using maltoheptaose as substrate and following the reaction with HPLC and ^{1}H-NMR spectroscopy, it was found that the action mode of the lyase followed a multichain attack mechanism. ^{1}H- and ^{13}C-NMR spectroscopic studies on unlabelled and labelled amylose (1-^{2}H, 2-^{2}H, 1-^{13}C) as substrates indicated that the lyase cleaved the C-(1')-O(4) bond forming a double bond between C-1' and C-2', thus forming the enol form of 1,5-anhydro-D-fructose. It also indicated that the catalytic process of the lyase involved proton exchanges among C-1, C-2, C-3 and the solvent.

Keywords: α-1,4-Glucan lyase; 1,5-Anhydro-D-fructose; Starch; Glycogen

1. Introduction

Starch, glycogen and related oligosaccharides are of prime importance in the living process and life cycle of organisms. Their accumulation and subsequent utilization comprise the fundamental metabolic activities of living cells. This has been the research subject for over a century [1]. In the degradation of these glucose polymers, the research interests have been focused on the already known α-glucan degrading enzymes, i.e. starch hydrolases and starch phosphorylases and the re-partitioning of the degrading products glucose and glucose 1-phosphate to other metabolic pathways, as well as the regulation of the whole process (for review see [2–4]).

In the study of AF, the precursor of microthecin, an antibiotic in morcels and other fungi, Baute et al. [5] found that glycogen was the precursor for AF and subsequently they demonstrated an enzyme activity that was able to convert glycogen to AF. However, it was not clear whether the production of AF from glycogen was catalysed by one or more enzymes and due to the presence of other α-glucan degrading enzymes in the enzyme preparation, an accurate characterization of the enzymes involved was not possible.

In the study of starch metabolism in red seaweeds, we succeeded in purifying an α-glucan degrading enzyme that produces AF [6]. Subsequently we showed the chloroplast location of this enzyme in the red seaweed *Gracilariopsis lemaneiformis* [7]. The present investigation examines the substrate specificity, action mode, and cleavage mechanism of the algal lyase.

Abbreviations: AF, 1,5-anhydro-D-fructose; BPNPG7; *p*-nitrophenyl-(4,6-*O*-benzylidene)-α-maltoheptaoside; G1, G2, G3 and so on, represents glucose, maltose, maltotriose, *etc.*; Mops, 3-[*N*-morpholino]propanesulfonic acid; PNPG1, *p*-nitrophenyl α-D-glucoside; PNPG2, *p*-nitrophenyl α-maltoside; PNPG7, *p*-nitrophenyl α-maltoheptaoside.

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2. Materials and methods

2.1. Chemicals

Maltose was purchased from Fluka (Switzerland). Maltotriose, maltotetraose, maltopentaose, maltohexaose, maltoheptaose, panose, nigerose, isomaltose, cyclohexaamylose, amylose, amylopectin, glycogen, *p*-nitrophenyl α-D-galactopyranoside, *p*-nitrophenyl α-D-mannopyranoside and rabbit muscle phosphorylase *b* were bought from Sigma (USA). D-Glucose (1-^{2}H, 2-^{2}H, 1-^{13}C, respectively) was obtained from Omicron Biochemicals (USA), methyl α-D-glucopyranoside from United States Biochemicals, maltitol from Serva (Germany), BPNPG7 from MegaZyme (Australia), and PNPG1, PNPG2 and PNPG7 from Boehringer Mannheim (Germany). Soluble starch and HPTLC silica gel 60 aluminium sheets were bought from Merck (Germany), and Termamyl from Novo Nordisk (Denmark). Sulfated galactans were purified from *Gracilaria chilensis* [8]. All the other reagents used were of the highest quality available. Dialysis tubes, with a molecular mass cut-off of 3500 Da, were obtained from Spectrum Medical (USA).

2.2. General methods

Thin-layer chromatography was performed by using HPTLC sheets pre-developed with methanol. Ethyl acetate/acetic acid/methanol/water (12:3:3:2 by volume) was used as the mobile phase. Double developments were performed upwards at room temperature. The products were detected using the standard spray reagent anisaldehyde-sulfuric acid (acetic acid/H_2SO_4/anisaldehyde, 100:2:1 by volume). The TLC method developed here was efficient in separating AF, glucose and maltosaccharides from maltose through maltoheptaose.

NMR spectra were recorded with a Varian VXR-400 instrument at a probe temperature of 25° C or 30° C. Samples (2–10 mg) were dissolved in 0.7 ml ^2H_2O. ^{1}H-NMR spectra were recorded at 400 MHz and the chemical shifts were referenced to internal sodium 3-trimethylsilylpropionate (TSP, δ 0.00). ^{13}C-NMR spectra were recorded at 100 MHz and referenced to internal 1,4-dioxane (δ 67.4). For complete assignment of signals DEPT and different H,H- and C,H-COSY experiments were performed, using pulse sequences available in the standard Varian software.

Matrix-assisted laser-desorption mass spectrometry was performed in the positive mode on an LDI-1700XS time-of-flight instrument using sinapinic acid as matrix and rabbit muscle phosphorylase *b* with a molecular mass of 97412 Da as a reference.

2.3. Purification of the α-1,4-glucan lyase and assay of its activity

α-1,4-Glucan lyase was purified from the red seaweed *G. lemaneiformis* (Bory) Dawson, Acleto et Foldvik as described earlier [6]. The major purification steps involved affinity chromatography on a column of starch followed by ion-exchange chromatography on Q-Sepharose and gel filtration on Sephacryl S-200. The measurement of α-1,4-glucan lyase activity was based on the production of AF as described earlier [6]. The standard reaction mixture (0.3 ml) contained the algal lyase in 50 mM Mops-NaOH (pH 6.2). The reaction was performed at 30° C and stopped by heating the samples in a boiling water bath for 2 min. For maltosaccharides, glucose polymer substrates, and for lactose, sucrose, trehalose and melibiose, the final concentration was 10 mg ml^{-1} or as specified in the text. For nigerose, panose, maltitol, isomaltose, cellubiose and pullulan, the concentration was 4 mg ml^{-1}. For substrates of PNPG1, PNPG2, PNPG7, BPNPG7, *p*-nitrophenyl α-D-galactoside and *p*-nitrophenyl α-D-mannoside, the final concentration was 2 mM. For methyl α-D-glucoside and cyclohexaamylose, the concentration was 10 and 20 mM, respectively. To compare the degradation rates in water and ^2H_2O, the lyase and the substrates maltose and amylopectin were lyophilized three times in the respective solvents before the reactions were started. Certain lyase-catalysed reactions were also monitored by HPLC, HPTLC and ^{1}H-NMR spectroscopy as specified in the text. The concentrations of the substrates were chosen such that they would not limit the reaction rate in the reaction rate studies. In the case of inhibition studies, methyl α-D-glucoside or cyclohexaamylose at a final concentration of 4 mM was included in the standard reaction mixture.

2.4. Synthesis of isotope-labelled amylose (1-^{2}H, 2-^{2}H, 1-^{13}C)

Labelled α-D-glucose 1-phosphate was synthesized from labelled D-glucose (1-^{2}H, 2-^{2}H, 1-^{13}C, respectively) according to MacDonald [9]. Labelled amylose was synthesized by using the differently labelled α-D-glucose 1-phosphate, potato phosphorylase and maltoheptaose as primer [10,11]. The conversion of α-glucose 1-phosphate to amylose was monitored by the disappearance of α-D-glucose 1-phosphate in the reaction system enzymatically [12] and by the formation of a blue complex with iodine. The structure of synthesized amylose was confirmed by ^{1}H-NMR spectroscopy; amylose-1-^{2}H, no signal for anomeric protons, amylose-2-^{2}H, singlet for H-1 at δ 5.40, amylose-1-^{13}C, a signal for the anomeric proton with the ^{1}H-^{13}C one-bond coupling constant $^1J_{C,H}$ at 169 Hz.

2.5. Preparation of 6^2-α-maltosylmaltotriose and its degradation by the lyase

Glycogen (8 g) in water (40 ml) was treated at 60° C for 18 h with Termamyl (100 μl), an endoamylase produced by *Bacillus licheniformis* which hydrolyses the α-1,4-linkage to form soluble dextrins and oligosaccharides. 1 g of the products was separated on a column (90 × 6 cm) of Sephadex G-25 using water as eluent. The eluate was monitored by differential refractometry. The main product

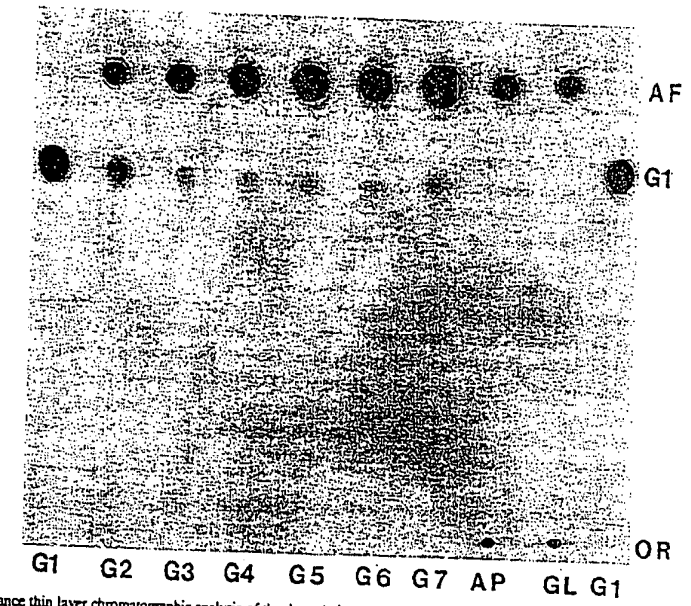


Fig. 1. High performance thin layer chromatographic analysis of the degradation products of maltosaccharides, amylopectin and glycogen by the lyase. The reaction mixture contained 0.3 ml 50 mM Mops-NaOH (pH 6.2), the lyase (0.37 U) and the various substrates in a final concentration of 1% (w/v). The reaction was carried out at 30° C for 24 h and stopped by heating for 2 min in a boiling water bath, and then analyzed by HPTLC. G1, glucose, G2, maltose, G3, maltotriose and so on. AP and GL were amylopectin and glycogen, respectively. OR, origin.

was found to be the branched pentasaccharide 6^2-α-maltosylmaltotriose. This pentasaccharide (3 mg) in ^2H_2O (0.7 ml) was treated with the lyase (0.16 U) at 30° C for 6 h and then another portion of the lyase (0.16 U) was added. The reaction was continued for 18 h and monitored by ^{1}H-NMR spectroscopy. The chemical structures of 6^2-α-maltosylmaltotriose and its degradation product, the branched tetrasaccharide 6^2-α-D-glucosylmaltotriose were verified according to methylation analysis, ^{1}H-NMR spectroscopy [13] and matrix-assisted laser-desorption mass spectrometry.

2.6. Production of 1,5-anhydro-D-fructose

Amylopectin (2 g) was dissolved in distilled water (100 ml) with the lyase added. The mixture was transferred to a dialysis tube and dialysed against distilled water at 30° C for 48 h (3 × 400 ml). The dialysate was freeze-dried. The authenticity and purity of the AF were confirmed by ^{1}H-NMR spectroscopy: δ 3.46 (H-1a, d, *J* 11.9), 3.75 (H-1b, d, *J* 11.9), 3.56 (H-3, d, *J* 8.8), 3.44 (H-4, dd, *J* 8.8), 3.39 (H-5, m), 3.68 (H-6, dd, *J* 6.6, 12.4), 3.90 (H-6', dd, *J* 2.1, 12.4), and by ^{13}C-NMR spectroscopy: δ 93.3 (C-2), 83.3 (C-5), 79.6 (C-3), 74.4 (C-1), 71.7 (C-4), 63.9 (C-6).

2.7. Separation and quantification of AF, glucose and maltosaccharides

Separation and quantification of AF, glucose and maltosaccharides were achieved by HPLC on a Dionex anion-exchange chromatography system equipped with pulsed amperometric detection (Series 4500i). The column used was CarboPac™ PA1 (4 × 250 mm). For the separation of AF and glucose, the column was eluted with 0.2 M NaOH. For the separation of maltosaccharides, the column was first eluted with 0.1 M NaOH for 1 min and then with a 1 M sodium acetate gradient (100% after 85 min). The detector response for each saccharide was determined by a known mixture of authentic saccharides.

To investigate the product ratio of AF to glucose, the lyase (0.37 U) was added to each of seven tubes containing 0.3 ml Mops-NaOH (50 mM, pH 6.2) and 3 mg of glucose, maltose through maltoheptaose, respectively. The reaction was carried out at 30° C for 24 h and stopped by heating the samples for 2 min in a boiling water bath. Samples were taken for AF and glucose analysis. To examine the time-course of the degradation of maltoheptaose, the lyase (2.87 U) was added to a 2% (w/v) maltoheptaose solution (4 ml). The reaction was carried out at 30° C. At 0, 2, 10, 20, 60 and 300 min, samples

were taken and boiled for 2 min before being analysed for AF, glucose and maltosaccharides.

In a similar experiment, the time-course degradation of maltoheptaose was followed by ^{1}H-NMR spectroscopy. Maltoheptaose was dissolved in ^2H_2O and sodium 3-trimethylsilylpropionate (0.5 mg) was added as an internal standard. ^{1}H-NMR spectra were recorded after 0, 0.5, 2, 4, 6 and 24 h. The spectra for 0 and 24 h are given in Fig. 3.

For calculation of the change in the proportion of α-1,6- to α-1,4-linkage, glycogen or amylopectin (20 mg) in ^2H_2O (0.7 ml) was incubated with the lyase (0.08 U) at 30° C for 18 h and the reaction was followed by ^{1}H-NMR spectroscopy. The proportion was calculated based on the intensity of the signals from the anomeric protons of the α-1,6- and α-1,4-linked glucose units.

2.8. Reaction mechanism studies

Amylose, [1-^{2}H]- and [2-^{2}H]amylose (20 mg each) were separately treated with the lyase in H_2O or ^2H_2O. When the enzyme reactions were to be carried out in ^2H_2O, the different amyloses and the lyase were first freeze-dried from a ^2H_2O solution for 3 times. The positions and yields of the deuterium in the formed AF were obtained by comparison of the integrals of the different proton signals in the ^{1}H-NMR spectra. The reduction of the signal intensities corresponded to the amount of incorporated deuterium.

3. Results

3.1. The molecular mass of the algal lyase

The purified α-1,4-glucan lyase from the red alga *G. lemaneiformis* is a single polypeptide with a molecular mass of 111 000 Da estimated by SDS-PAGE [6]. Considering the greater deviation of ±10% by the SDS-PAGE method, the molecular mass was re-estimated to be 116 654 (±500 or ±0.4%) Da by matrix-assisted laser-desorption mass spectrometry in this study.

3.2. The products of the lyase catalysed reaction

The degradation of various substrates by the algal lyase was followed by HPTLC, HPLC on a Dionex system and ^{1}H-NMR spectroscopy. For linear maltosaccharides, a complete degradation was achieved as only two spots on the HPTLC plate were detected, AF (the fast moving spot) and glucose (the slow moving spot) (Fig. 1). With glucose as the substrate, no AF was formed (Fig. 1). The quantitative analysis of the degradation products of linear maltosaccharides, AF and glucose is given in Table 1. It can be noted that the relative amount of AF increased while that of glucose decreased with increasing size of the maltosaccharide. When maltose was used as substrate, the ratio of AF to glucose was 1:1, and in the case of maltoheptaose the ratio was 6:1 (Table 1). The branched pentasaccharide (6^2-α-maltosylmaltotriose) was degraded to AF and the branched tetrasaccharide (6^2-α-D-glucosylmaltotriose) with a product ratio of 1:1 (Fig. 2). At a very slow rate, panose (6^2-α-D-glucosylmaltose) was degraded to AF and glucose with an AF/glucose ratio of 2:1. The degradation products of PNPG1, PNPG2 and PNPG7 were AF and *p*-nitrophenol.

Table 1
The relative yields of 1,5-anhydro-D-fructose and glucose after the complete degradation of the maltosaccharides

Substrates:	G1	G2	G3	G4	G5	G6	G7
Products:							
Glc yield (%)	100	50	33	25	20	17	14
AF yield (%)	0	50	66	74	80	84	87
AF/Glc	0	1	2	3	4	5	6

The reaction conditions were the same as in Fig. 1. AF and glucose were quantified by HPLC on a Dionex system using authentic AF and glucose as standards.

In the case of amylopectin and glycogen, no glucose or other side reaction products were detected and AF was the only migrating product on the HPTLC plate (Fig. 1). For amylopectin, the proportion of α-1,6 to α-1,4-linkages was 5.2% at the start of the enzymic reaction and increased to 9.6% at the end of the reaction. In the case of glycogen, the proportion increased from 12.5% to 16.0%.

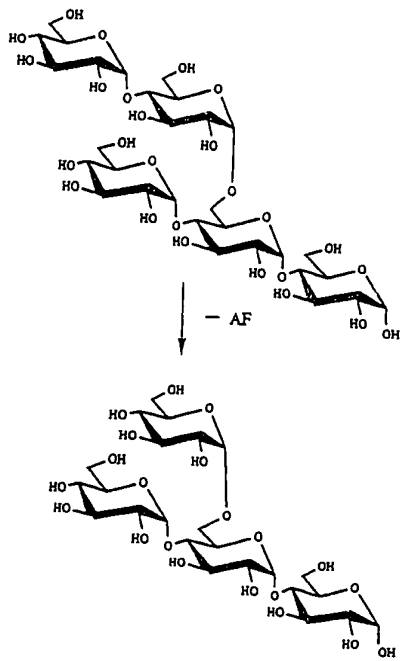


Fig. 2. Degradation of the branched pentasaccharide 6^2-α-maltosylmaltotriose to the branched tetrasaccharide 6^2-α-D-glucosylmaltotriose by the lyase. The substrate (3 mg) in ^2H_2O (0.7 ml) was treated with the lyase (0.16 U) at 30° C. A complete degradation was achieved after 6 h as monitored by ^{1}H-NMR spectroscopy.

Table 2
Release of 1,5-anhydro-D-fructose from maltosaccharides and α-glucans by the lyase

Substrates	AF released (nmol min^{-1})
Maltose	98.6
Maltotriose	57.8
Maltotetraose	41.1
Maltopentaose	54.1
Maltohexaose	110.3
Maltoheptaose	147.6
Glycogen	114.8
Amylopectin	139.2
Amylose	148.5
Soluble starch	147.9
Floridean starch	91.6

The reaction mixture contained the substrate (3 mg) and the lyase (0.15 U) in 50 mM Mops-NaOH (pH 6.2, 0.3 ml). The reaction was carried out at 30° C for 15 min and stopped by heating for 2 min in a boiling water bath.

3.3. Substrate specificity and the degradation of the lyase towards various substrates

The enzyme was examined with respect to its ability to degrade a variety of substrates (Table 2). It formed AF from maltoheptaose at a similar rate as that observed from amylopectin, amylose, and soluble starch. The activity was slightly lower with glycogen and floridean starch. The lyase showed very low activity towards nigerose and panose. Under the same reaction conditions listed in Table 2, the production of AF from nigerose and panose was not detectable. When the incubation time was prolonged to 24 h, however, both nigerose (Glc $p\alpha1 \rightarrow 3$Glc) and panose were completely degraded to AF and glucose. At such long term incubation 4% of maltitol (Glc $p\alpha1 \rightarrow 4$Glucitol) and trace amount of BPNPG7, the non-reducing end blocked *p*-nitrophenyl maltoheptaoside (less than 1%) were degraded.

6^2-α-Maltosylmaltotriose was completely degraded to 6^2-α-D-glucosylmaltotriose with the release of AF after 6 h in the presence of 0.16 units of lyase (Fig. 2). No further degradation of the branched tetrasaccharide occurred even with another addition of the lyase (0.16 U) and prolonging the incubation to 18 h as indicated by the ^{1}H-NMR spectrum.

Table 3
Time-course degradation of maltoheptaose by the lyase as monitored by HPLC

Reaction time (min):	0	2	10	20	60	300
Products:						
AF	–[a]	25.7	55.2	96.0	216.0	346.8
G1	–	–	–	–	21.1	57.8
G2	–	–	3.8	11.0	5.3	–
G3	–	–	–	–	7.0	–
G4	–	–	1.9	3.3	5.3	–
G5	–	1.6	6.2	7.1	4.9	–
G6	–	22.5	18.1	16.9	8.2	–
G7	57.8	33.7	27.7	18.7	6.0	–

The reaction mixture in water (4 ml) contained the lyase (2.87 U) and maltoheptaose in a final concentration of 2% (w/v). The reaction was carried out at 30° C. At 0, 2, 10, 20, 60, and 300 min, samples (0.3 ml) were taken and heated for 2 min in a boiling water bath to stop the reaction. The occurrence of malto-oligosaccharides products and the final products, AF and glucose, were quantified by chromatography on a Dionex system. The data are presented in nmol of each sugar produced at the respective reaction intervals.
[a], not detectable.

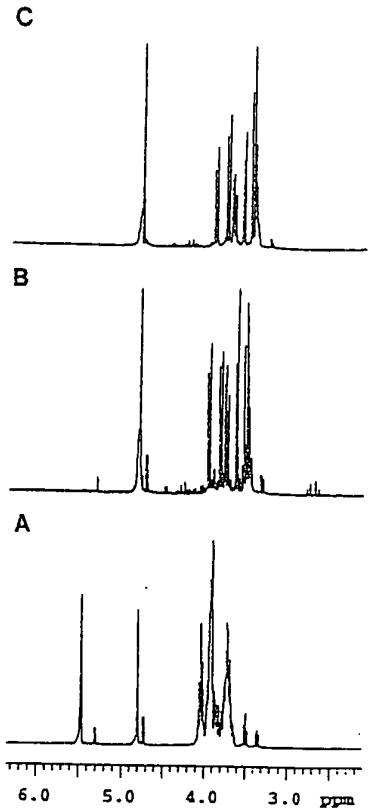


Fig. 3. The degradation of maltoheptaose as monitored by ^{1}H-NMR spectroscopy. Maltoheptaose (9 mg) was dissolved in 0.7 ml ^2H_2O containing sodium 3-trimethylsilylpropionate (0.5 mg) as an internal standard. The reaction was started by the addition of 1.10 U of lyase. A, the ^{1}H-NMR spectrum at the start of the reaction. B, the spectrum after 24 h incubation. C, the spectrum of AF.

Among the α-D-glucosides, methyl α-D-glucoside was not degraded; PNPG1 was the smallest substrate that was degraded; PNPG2 and PNPG7 were at least as active as maltose and maltoheptaose, respectively. As with glucose (Fig. 1), the lyase was not able to convert other monosaccharides, such as fructose, galactose and mannose to AF or to any other product. No AF or glucose were released as checked by HPTLC after 24 h of incubation with isomaltose (Glc $p\alpha 1 \rightarrow 6$Glc), trehalose (Glc $p\alpha 1 \rightarrow 1\alpha$Glc p), cellubiose (Glc $p\beta 1 \rightarrow$ 4Glc), sucrose (Glc $p\alpha 1 \rightarrow 2\beta$ Fru f), melibiose (Gal $p\alpha 1 \rightarrow$ 6Glc), lactose (Gal $p\beta 1 \rightarrow$ 4Glc), p-nitrophenyl α-D-galactoside, p-nitrophenyl α-D-mannoside, and cyclohexaamylose. The algal α-1,4-glucan lyase did not show any detectable activity towards pullulan or sulfated galactans, a red algal cell wall component (the cold water extract of an agar fraction purified from *Gracilaria chilensis* [8]. At a concentration of 4 mM, cyclohexaamylose and methyl α-D-glucoside did not show any inhibitory effect on the catalytic activity of the lyase, when maltose or soluble starch was used as substrate.

3.4. Mode of action of the lyase

A time-course of the degradation of maltoheptaose by lyase action was followed by HPLC on a Dionex system (Table 3), ^{1}H-NMR spectroscopy (Fig. 3), and HPTLC (figure not shown). During the progress of reaction, besides AF, the other products were found to be a mixture of di- to hexa-saccharides. The maltosaccharide nature of these was further confirmed by ^{1}H-NMR spectroscopy. As shown in Table 3, already after 2 min of incubation, more than one third of the maltoheptaose was converted to maltohexaose, AF and a small amount of maltopentaose, while glucose was detected only after 60 min of incubation. The whole reaction was complete after 5 h as the product ratio of AF to glucose reached a value of 6:1 (Table 3).

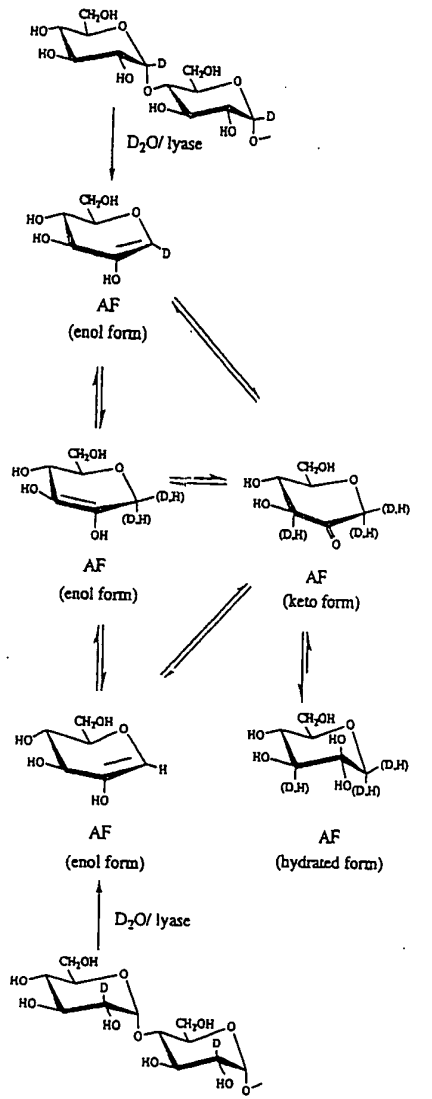
3.5. Studies of the mechanism for cleavage of α-1,4-glucosidic linkage

Using potato phosphorylase [1-^{2}H]-, [2-^{2}H]- and [1-^{13}C]amylose were synthesized from differently labelled α-D-glucose 1-phosphate (1-^{2}H, 2-^{2}H and 1-^{13}C). These labelled amyloses were treated with lyase in both H_2O and ^2H_2O, respectively, and the AF obtained from these reactions was analysed by ^{1}H-NMR spectroscopy with respect to the amounts and positions of the deuterium. The integrals of the different signals were compared to those of the H-6 signals, as these signals showed no overlap.

In the AF obtained from treatment of unlabelled amylose with lyase in ^2H_2O as reaction medium, the two coupled signals from H-1a and H-1b, an intact -CH_2- group, had 70% intensity and the H-3 signal 90%.

In the AF obtained from treatment of [1-^{2}H]amylose with lyase in H_2O as reaction medium, only half of the deuterium remained in the 1-position, as the coupled H-1a and H-1b signals had only 50% intensity. Using ^2H_2O as reaction medium the two signals from H-1a and H-1b had further decreased to only 15% intensity and the H-3 signal to 50%.

In the AF obtained from lyase treatment of [2-^{2}H]amylose in H_2O as reaction medium, the coupled H-1a and H-1b signals had 30% intensity and the H-3 signal



Scheme 1. The cleavage mechanism of the lyase-catalyzed reaction and the interconversion of different forms of AF.

75%. Using ^2H_2O as reaction medium the two signals from H-1a and H-1b had 20% intensity and the H-3 signal 50%.

When unlabelled AF was dissolved in ^2H_2O the signals of H-1 and H-3 showed no decrease in intensity even after 1 wk. The results given above therefore indicate that all the observed deuteron/proton exchanges among C-1, C-2, C-3 and the solvent occurred during the enzyme reaction and not after the AF had been formed.

The results suggested that the protons on C-1 of AF could be derived both from the reaction medium and H-1, H-2 and H-3 of the original glucose unit. This was indicated by: (i) the incorporation of deuterium from the solvent to C-1 and C-3 by 30% and 10%, respectively, in AF obtained from reaction with unlabelled amylose in ^2H_2O; (ii) the incorporation to approximately 15% of two hydrogens at C-1 in AF when [1-^{2}H]amylose was used as substrate and ^2H_2O as the reaction medium, showing that part of H-2 (or H-3) were transferred to the 1-position; (iii) the incorporation of deuterium to C-1 to approx. 80% when [2-^{2}H]amylose was used as substrate and ^2H_2O as the reaction medium.

When [1-^{13}C]amylose was used as substrate, a ^{13}C-NMR spectrum showed that AF was labelled at C-1, the same position as the original glucose unit. Neither signals for a carbonyl carbon nor an alkene group (> C = C <) were detected.

The reaction rate decreased by 30% when the reaction was performed in ^2H_2O as compared to that observed in water. This was the case when maltose or amylopectin was used as substrate.

4. Discussion

The present work forms the basis for an understanding of the α-1,4-glucan lyase-catalysed reaction. The final degradation products of the lyase-catalysed reaction are AF, glucose or a limit dextrin, depending on whether the substrate is a linear or a branched glucan. This is in sharp contrast to the reaction pattern of other starch degrading enzymes. Thus with starch hydrolases, the degradation products are a series of maltosaccharides and glucose; with starch phosphorylase, the degradation product is glucose 1-phosphate [12]. The reaction catalysed by the lyase and the subsequent metabolism of AF therefore represents a new, alternative starch/glycogen degrading pathway, "The Anhydrofructose Pathway", to be distinguished from the well known hydrolytic and phosphorolytic pathways [3].

The algal lyase readily cleaved α-1,4-D-glucosidic bonds in maltosaccharides and branched α-glucans with the formation of AF (Table 2). It was, however, not able to cleave the α-1,4-D-glucosidic bond at the 4-position of the 4,6-branched residue as in the case of 6^2-α-D-glucosylmaltotriose (Fig. 2). In general, the lyase was not able to degrade α-1,6-glucosidic bonds, as it showed no activity towards isomaltose and 6^2-α-D-glucosylmaltotriose. On addition of pullulanase that hydrolyses terminal α-1,6-linkages, the yield of AF increased by 18–27% when branched α-glucans were used as substrate (Yu et al., unpublished data). As an exception to this rule, the α-1,6-linkage in panose was, however, cleaved by the lyase with the formation of AF and glucose in a proportion of 2:1, though at a very slow rate. This suggests that in the degradation of panose the α-1,6-linkage was first broken with the formation of AF and maltose as the intermediate product and this was the rate-limiting step. The maltose formed will quickly be converted to AF and glucose. More information concerning the enzyme reaction site is needed, however, to explain why the lyase can cleave the α-1,6-linkage in panose but not in isomaltose, 6^2-α-D-glucosylmaltotriose and branched α-glucans.

The ability of the lyase to degrade α-1,3-glucosidic bonds is limited as was shown from its low activity towards nigerose. The lyase was not capable of cleaving the α-1,1-bonds in trehalose. No activity was detected towards α-1,4-linkage of other α-glycosides, such as p-nitrophenyl α-D-mannoside and p-nitrophenyl α-D-galactoside, with axial hydroxyls at C-2 (mannose) or at C-4 (galactose), respectively. From these substrate specificity studies, it can therefore be concluded that the algal lyase is highly specific for α-1,4-D-glucosidic bonds.

The inability of the algal α-1,4-glucan lyase to degrade cyclohexaamylose, pullulan and the extremely low activity towards BPNPG7 indicate that it is an exo-lyase and has little or no endo-cleavage activity. BPNPG7 is a p-nitrophenyl maltoheptaoside with its non-reducing end substituted with a benzylidene group at the 4,6-position, a substrate specially designed for the determination of endoamylolytic activity [14]. The incapability of the enzyme to degrade BPNPG7 is not due to the p-nitrophenyl group, as PNPG7 was as active as maltoheptaose. This result supports the notion that the lyase starts its lytic action from the non-reducing end of an α-glucan. This action mode is consistent with all other known exo-α-glucan depolymerizing enzymes [3] and also agrees with the molecular structure of amylopectin.

From the relationship between the size of the maltosaccharides and the ratio of AF to glucose formed in the reaction (Table 1), and from the fact that the lyase was not able to convert monosaccharides like glucose to AF, it is deduced that the lyase removes glucose units successively as AF from an α-1,4-glucan chain until the last glucose unit on the reducing end is left. This is the case when a linear glucan is used as substrate. In a comparative case, α-1,4-glucan phosphorylase starts its degradation from the non-reducing end until 4 glucose units are left at the reducing end [3].

The relative low activity of the algal lyase towards glycogen may be related to its higher branching or shorter lengths of side chains than amylopectin. The amylopectin

used in this study had a proportion of α-1,6 to α-1,4-linkages of 5.2%, while that for glycogen was 12.5%. The algal α-1,4-glucan lyase readily degraded heat-denatured floridean starch, isolated from the same plant from which the enzyme was purified (Table 2). The rate was, however, lower than that observed with soluble starch and amylopectin.

When a linear glucan like maltoheptaose was used as substrate, besides AF, the reaction intermediate products were a pool of different maltosaccharides (Table 3). An analysis of the time course of the appearing and disappearing of these maltosaccharides provided valuable information on the action mode of the lyase. Degradation of soluble polysaccharides by enzymes usually proceeds by three different mechanisms: single chain attack, multichain attack and multiple attack [3,15]. Which mechanism that prevails depends on the specific enzyme. The algal α-glucan lyase proceeds obviously via a multichain attack mechanism. That is, after the initial lytic cleavage of the α-1,4-linkage, the maltosaccharide product is released from the enzyme and for subsequent cleavage, the enzyme attacks all the substrate molecules in a random fashion. Thus a significant amount of maltohexaose was formed in the initial reaction period, whereas glucose was only detected at latter stage (Table 3).

The results from the degradation of unlabelled and labelled amylose in H_2O and ^2H_2O indicate that a double bond between C-1 and C-2 was formed during the cleavage of the α-1,4-linkage, yielding the enol form of AF (Scheme 1). The occurrence of the enol form was verified by the incorporation of deuterium to C-1 of AF when the enzymic reaction was performed in ^2H_2O using unlabelled amylose as substrate. The decrease of the signal for H-3, resulting from the incorporation of deuterium to C-3, implied that the enol form also existed with C-3 (Scheme 1). As little or no exchange occurred for the protons at C-1 and C-3 when AF was kept in ^2H_2O even in the presence of the lyase, it is suggested that the exchange of these protons takes place during the formation of AF in the enzyme reaction site. Further information is needed concerning the roles of the specific amino acid residues of the lyase in the catalysis process.

In an aqueous solution of AF, the equilibrium between these different forms lies far towards the hydrated form (Scheme 1), as only the signal at δ 93.3 ppm for the hydrated C-2 was observed and neither that for the $>C=C<$ nor the $>C=O$ group was detected in the ^{13}C-NMR spectrum.

In the lyase catalysed reaction, the additional proton on C-1 of the AF can be derived from the aqueous reaction medium and the proton on C-2 of the glucose unit. In accordance with this the highest incorporation of deuterium to C-1 of AF was obtained when deuterium was present in both the reaction medium and in the substrate.

It seems that water to some extent is involved in the rate limiting step of the lyase-catalysed reaction since the rate decreased by 30% when ^2H_2O was used instead of H_2O as the reaction medium.

5. The classification characters of the lyase

i. The recommended name for the algal lyase: α-1,4-glucan lyase; α-1,4-glucan 1,5-anhydro-D-fructose eliminase; α-1,4-glucan exo-lyase.

ii. The systematic name: α-1,4-glucan exo-4-lyase (1,5-anhydro-D-fructose-forming), (EC 4.2.2.-).

The naming of this enzyme was according to the recommended rules set by the Nomenclature Committee of the International Union of Biochemistry and Molecular Biology (NC-IUBMB, [16]). The serial number will be given by the Committee.

iii. The reaction catalysed: cleavage of the terminal α-1,4-D-glucosidic bonds of an α-1,4-glucan successively from the non-reducing end of the chains with the release of 1,5-anhydro-D-fructose.

When a linear α-1,4-glucan, such as maltose, maltosaccharides and amylose, is used as a substrate, the reaction formula is:

Linear α-glucan → AF + Glc

The products are AF and glucose. The yield of AF (%) = $(n-1)/n \times 100$, The yield of glucose (%) = $1/n \times 100$. n indicates the number of glucose units in a linear α-1,4-glucan.

When a branched glucan (α-1,4-glucan with α-1,6-branches), such as amylopectin and glycogen, is used as substrate, the reaction formula is:

Branched α-glucan → AF + limit dextrin

The products are AF and the limit dextrin. The enzyme will release AF from the non-reducing ends and the degradation stops at glucosyl groups before the branched residue. Therefore the yields of AF and the limit dextrin depend on the percentage of the linear chain length with a non-reducing end.

iv. The biological sources known at present: the red seaweeds *G. lemaneiformis* and *G. verrucosa*.

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Reactions of 1,5-anhydro-D-fructose in alkaline aqueous solution

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Abstract

1',4-Anhydro-3-deoxy-2-*C*-(hydroxymethyl)-D-*threo*-pentonic acid [(3*R*,5*S*)-3-hydroxy-5-(hydroxymethyl)oxolane-3-carboxylic acid] has been isolated as the main product from treatment of 1,5-anhydro-D-fructose with aqueous NaOH at room temperature. The identification of the 4-deoxy-2,3-diulose and its two enol forms as reaction intermediates and the incorporation of deuterium in the products, when D_2O was used as solvent, provided evidence for the mechanism of the alkaline degradation. Further information has been obtained on the mechanism using 1,5-anhydro-(2-^{13}C)-D-fructose as starting material and on the stereochemistry of the final product by NOE measurements. The results showed exclusive migration of the 1,2-bond in the benzilic acid rearrangement of the 2,3-diulose, forming the product with the (*R*)-configuration at C-2.

The reaction of 1,5-anhydro-D-fructose with 3,5-dinitrosalicylic acid in aqueous NaOH at room temperature has also been studied. The main product was 1',4-anhydro-2-*C*-(hydroxymethyl)-D-arabonic acid.

Keywords: 1,5-Anhydro-D-fructose; Alkaline degradation; 1',4-Anhydrosaccharinic acid; (3*R*,5*S*)-3-Hydroxy-5-(hydroxymethyl)oxolane-3-carboxylic acid; 1',4-Anhydro-3-deoxy-2-*C*-(hydroxymethyl)-D-*threo*-pentonic acid

1. Introduction

The chemistry of 1,5-anhydro-D-fructose (AF) has recently become of increased interest, as it is the main product from the enzymatic degradation of α-1,4-glucans by an α-1,4-glucan lyase, isolated from red algae [1,2]. It has previously been found as a product from the degradation of starch by an enzyme extract from a strain of *Morchella vulgaris* [3]. AF has also been prepared by synthesis [4] and by the enzymatic conversion of 1,5-anhydro-D-glucitol [5].

The reactions and products formed during treatment of sugars with alkali have been extensively studied. α-Dicarbonyl compounds have been postulated as intermediates in the reactions, and these may undergo benzilic acid rearrangement to

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form saccharinic acids. Isbell early put forward a hypothesis for the reaction mechanism [6]. Later studies [7-10] on the alkaline degradation of substituted reducing sugars and maltose confirmed this hypothesis and resulted in the presentation of a general mechanism for the saccharinic acid formation. The formation of saccharinic acids and their acyclic fragments has been reviewed [11-12].

The present study was carried out to investigate the reactions of AF in aqueous alkaline solution. Information was obtained from the identification of the products and the reaction intermediates when AF or its ^{13}C-substituted analogue was used as reactant and H_2O or D_2O as solvent.

2. Experimental

Materials. – AF and (1-^{13}C)- and (2-^{13}C)-AF were prepared as previously described by the enzymatic degradation of amylopectin and of properly ^{13}C-substituted amylose using an α-1,4-glucan lyase isolated from red algae [2]. The structures of (1-^{13}C)- and (2-^{13}C)-AF were confirmed by ^1H and ^{13}C NMR spectroscopy.

Analytical methods. –TLC was performed on Merck aluminium precoated plates of Silica Gel $60F_{254}$ using $CHCl_3$-EtOH (7:3 or 17:3) as solvent, with detection by UV or by spraying with anisaldehyde-H_2SO_4 followed by heating at 100°C for 5 min.

For HPLC, a Merck C-18 LiChrospher (5 μm) column (125 x 4 mm) was used. The compounds were eluted with MilliQ-water at a flow rate of 1 mL min^{-1}. The effluent was monitored with a UV-detector at 280 nm.

FAB-mass spectra were recorded with a JEOL SX/SX102A instrument in the positive or the negative mode. Ions were produced by a beam of Xe atoms using glycerol/triethanolamine or *m*-nitrobenzyl alcohol as matrix.

NMR spectra were recorded at 400 (^1H) or 100.6 MHz (^{13}C) with a Varian VXR 400 instrument, a probe temperature of 25°C, and samples dissolved in $CDCl_3$ or D_2O. The chemical shifts were referenced to internal sodium 3-trimethylsilylpropionate-d_4 (TSP, δ_H 0.00) and *p*-dioxane (δ_C 67.40), respectively. For complete assignment of signals, H,H- and H,C-COSY and DEPT experiments were performed using pulse sequences available in the Varian software. NOE difference experiments on acetylated (**5a** and **6a**) and methylated (**5b** and **6b**) derivatives were recorded using $CDCl_3$ solutions degassed with He, and a preirradiation time of 10 s.

Alkaline degradation of AF. –(a) AF (20 mg) was dissolved in 1, 0.5, 0.1, 0.05 or 0.01 M NaOD in D_2O (0.7 mL), and the solutions were transferred to 5 mm NMR tubes. The reactions were followed by ^1H NMR spectroscopy, and the spectra were recorded after 5, 10, 15, 20, and 30 min and then after 24 h.

(b) AF (100 mg) was treated with aqueous NaOH (1 M, 3 mL) at room temperature for 10 min. The reaction mixture was neutralized with Dowex 50 (H^+) resin, filtered, and concentrated to a syrup. The main component **5** had formed in 95% yield as shown by a ^1H NMR spectrum. The product was analysed by FAB-MS and NMR spectroscopy.

(c) AF (20 mg) was treated with aqueous NaOH (0.5 M, 0.7 mL) at 25°C for 5 min followed by neutralization with HCl. The products were separated by HPLC and two fractions were collected and freeze-dried. The samples were dissolved in D_2O and analysed by NMR spectroscopy. The first fraction contained compound **4** and the

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second two compounds, **2** and **3**, in an approximate 3:2 ratio. The samples were analysed again after 24 h at room temperature.

Acetylation and methylation of 5. –Compound **5** (10 mg) was acetylated with Ac_2O (1 mL) in dry pyridine (1 mL) at room temperature overnight . The solution was poured into ice-water, and the resulting mixture extracted with $CHCl_3$. The extract was washed with 0.1 M HCl (2 x 25 mL), 0.1 M $NaHCO_3$ (2 x 5 mL), and water (2 x 2 mL), dried (Na_2SO_4), filtered, and concentrated to dryness yielding **5a** (13.6 mg, 90% yield).

Methylation was achieved by dissolving **5** (10 mg) in dry DMSO (1 mL) under nitrogen. 2 M $NaCH_2SOCH_3$ in DMSO (1 mL) was added. The solution was kept for 20 min in an ultrasonic bath, cooled in a freezer for 10 min, treated with cold methyl iodide (1 mL), and kept in an ultrasonic bath for 10 min. The product was isolated by a SepPac-C18 column yielding **5b** (10.5 mg, 84%).

Treatment of AF with alkali and 3,5-dinitrosalicylic acid (DNS). –DNS (20 mg) was dissolved in aqueous 1 M or 0.5 M NaOH (0.7 mL). AF (20 mg) was added to each solution, which was kept at room temperature for 10 min. The colour of the reaction mixture became dark yellow. Both solutions were neutralized with Dowex 50 (H^+) resin, filtered and concentrated to syrups. The main compound **6** was formed in 95% yield in both reactions as shown by ^1H NMR spectra.

Compounds **3** and **4** were treated with DNS dissolved in 1 M NaOD in D_2O as described above, and the mixture was analysed by ^1H NMR spectroscopy.

Compound **6** (10 mg) was acetylated and methylated as described above. The di-*O*-acetate **6a** was obtained in 88% yield (15.0 mg) and a methylated derivative, **6b**, as the main product according to the ^1H NMR spectrum.

3. Results and discussion

AF was obtained by the enzymatic degradation of amylopectin [2]. In aqueous solution, it occurs in the hydrate form (**1**), which was shown by FAB-MS, giving the $[M+Na]^+$ ion at *m/z* 203, the ^1H and ^{13}C NMR data (Tables 1 and 2) and the ^{13}C chemical shift differences (DIS) between the spectra in H_2O and D_2O solution [13]. AF was treated with different concentrations of NaOD in D_2O at room temperature, and the reactions were followed by ^1H NMR spectroscopy. At NaOD concentrations ≥ 0.05 M, the ^1H NMR spectrum of AF changed drastically, as all original signals vanished and instead, broad signals in the δ 3.3 - 3.8 region appeared (Fig. 1). When the solution was neutralized, the broad signals disappeared and those of AF returned, except that from H-3. H-3 is evidently replaced by deuterium from the solvent, D_2O, indicating that the anion, **1b**, of an enol form with the double bond between C-2 and C-3, is reversibly formed via the ketoform **1a** in the stronger alkali (Scheme 1). After 5 min, in 0.1 M NaOD, the intensities of these enol signals decreased and a new component, **2**, was formed according to the ^1H NMR spectrum (Fig. 2). This component contained a double bond, as evident from a signal at δ 5.60. After 10 min, the amount of **2** had increased and furthermore, signals from another unsaturated [redacted] rred as evident from a singlet at δ 7.07. Also signals in the region [redacted]y protons were observed (Fig. 2). On prolonged treatment, the [redacted]ginal enolate ion **1b** disappeared and component **2** was replaced [redacted] signals appeared indicating that another component, **5**, containing

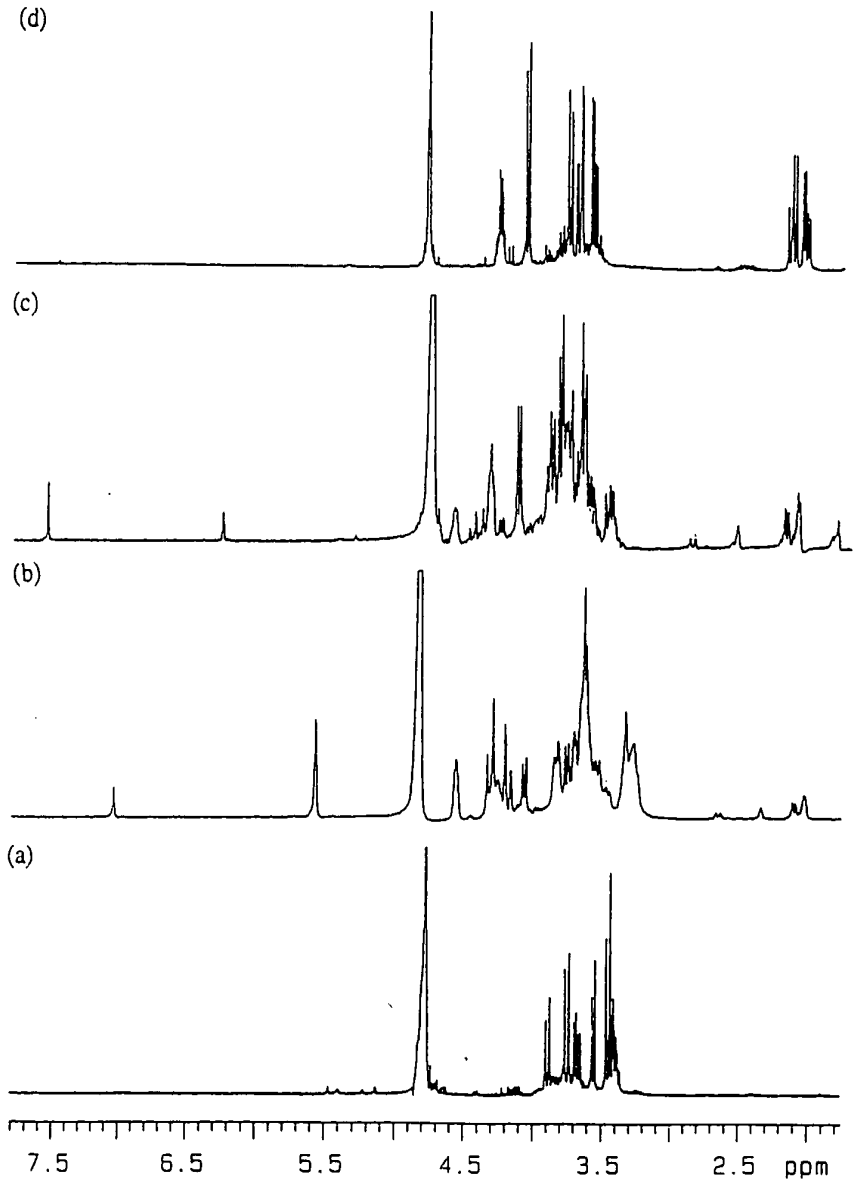
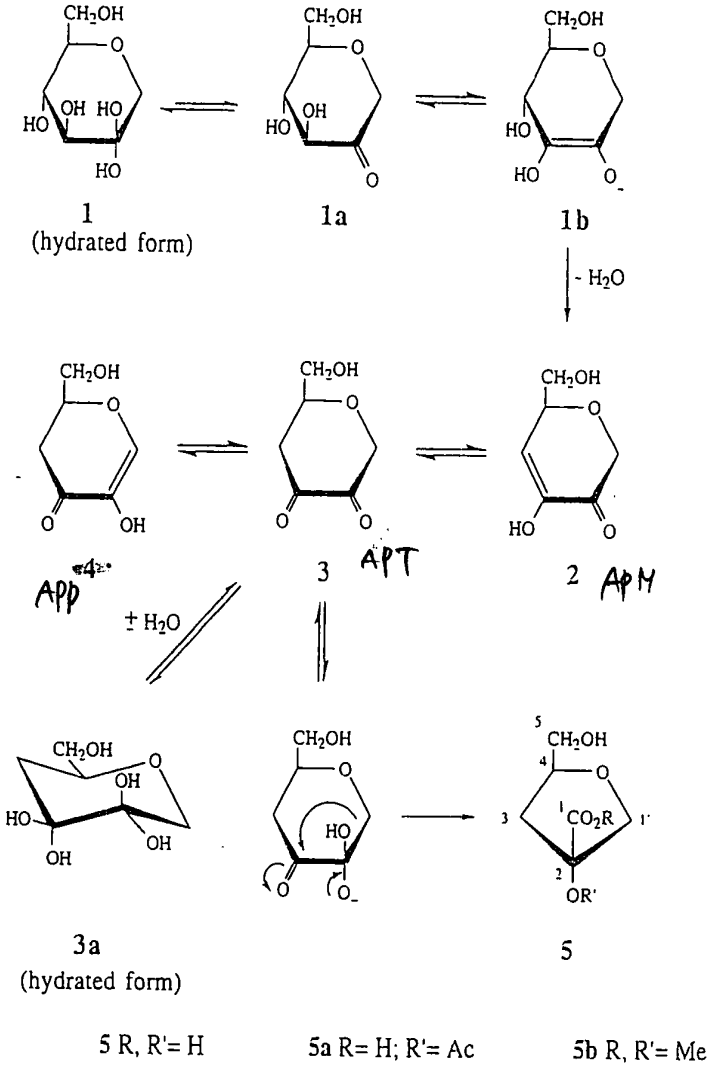


Fig. 1. 400-MHz ^{1}H-NMR spectra of (a) AF; (b) AF treated with 0.5 M NaOD in D_2O for 5 min; (c) The same as in (b) but after neutralization; (d) Saccharinic acid 5 obtained from treatment of AF with 1 M NaOH in H_2O at room temperature for 10 min.

deoxy protons was formed. If the reaction was allowed to continue, only signals from compound 5 were observed in the final reaction mixture (Fig. 1).

Using 0.5 M NaOD, the AF had disappeared after ≈10 min and according to the ^{1}H NMR spectrum, a mixture of 2 and 4 in the molar proportion 1:6 was obtained.



Scheme 1.

On prolonged treatment, with alkali compounds 2 and 4 rapidly decreased in amount and were completely replaced by 5 after 30 min. After neutralization, the chemical shifts for the signals from the olefinic proton of 2 and 4 changed to δ 6.23 and 7.55, respectively, indicating the conversion of the enolate form to the enol form (Fig. 1). Compounds 2-4 were partly separated from the neutralized reaction mixture by reversed phase HPLC. Compound 4 was isolated in pure form from the first fraction, whereas the second fraction contained a mixture of the two intermediates 2 and 3. After 24 h in a D_2O solution at room temperature, only compound 3 remained according to the NMR spectra. From the ^{1}H spin-systems and the ^{13}C chemical shifts

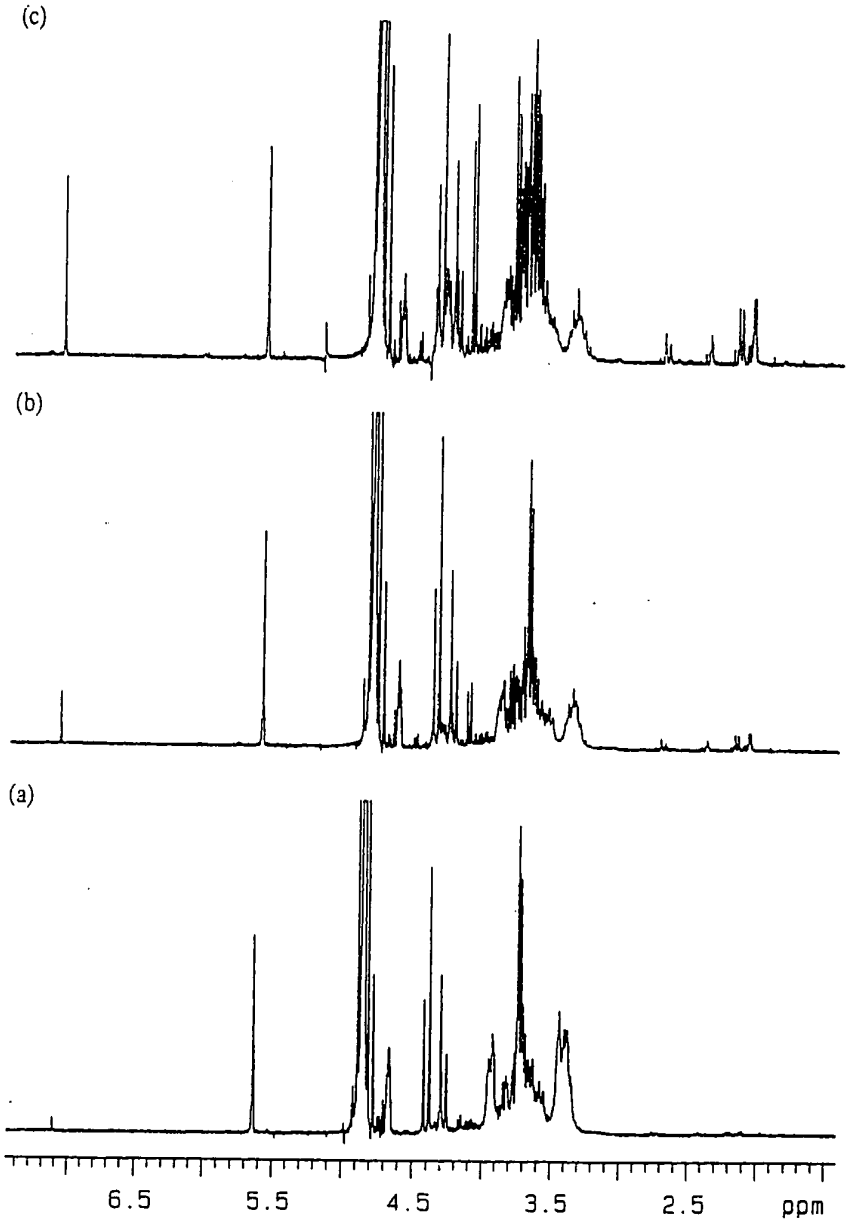


Fig. 2. 400-MHz ^{1}H-NMR spectra of AF treated with 0.1 M NaOD in D_2O for (a) 5 min, (b) 10 min, and (c) 20 min.

(Tables 1 and 2), assigned by DEPT and H,C-COSY experiments, the structures of 2-4 were determined. The position of the double bond between C-3 and C-4 in compound 2 was established from the ^{1}H NMR signal at δ 6.23, which was assigned to H-4 due to its coupling to H-5. Moreover, no H-3 signal could be detected. The coupling constant observed for the two protons on C-1 was 17.0 Hz, and such a large

Table 1

^{1}H and ^{13}C NMR chemical shifts (δ) for AF (1), reaction intermediates (2-4), 1',4-anhydrosaccharinic acid (5), and derivatives

Cpd	1	2	3a	4	5	5a [a]	5b [a]	6	6a [a]	6b [a]
Solv.	D_2O	D_2O	D_2O	D_2O	D_2O	$CDCl_3$	$CDCl_3$	D_2O	$CDCl_3$	$CDCl_3$
H-1(a)	3.46	4.36	3.63	7.55						
H-1b	3.75	4.46	3.69							
C-1	74.4	73.35	73.20	154.09	179.91			183.07		
H-1'a					3.77	4.02	3.99	3.42	4.11	4.09
H-1'b					4.08	4.51	4.15	3.91	4.60	4.25
C-1'					78.59			79.35		
C-2	93.3		95.31	137.44	83.66			87.09		
H-3(a)	3.56				2.04	2.33	2.16	3.81	5.53	4.05
H-3b					2.14	2.43	2.26			
C-3	79.6	148.84	96.25	194.09	41.46			84.13		
H-4(a)	3.44	6.23	1.81	2.85	4.27	4.35	4.30	3.30	4.12	4.00
H-4b			1.86	2.88						
C-4	71.7	122.94	39.80	39.31	80.84			80.49		
H-5(a)	3.39	4.71	3.82	4.58	3.70	4.14	3.30	3.32	4.28	3.52
H-5b					3.59	4.27	3.48	3.52	4.44	3.62
C-5	83.3	78.06	78.73	82.28	64.03			64.95		
H-6a	3.68	3.77	3.59	3.79						
H-6b	3.90	3.82	3.66	3.89						
C-6	63.9	65.92	66.61	65.14						

[a] In the ^{1}H NMR spectra of 5a and 6a, the AcO groups appeared at δ 2.10, 2.13 and 2.07, 2.08, 2.15, respectively. In those of 5b and 6b, the MeO groups appeared at δ 3.31, 3.39, 3.78 and 3.41, 3.44, 3.48, 3.82, respectively.

value implies that these protons are adjacent to a carbonyl group. A long-range coupling (^4J 2.1 Hz) between H-1b and H-5 indicated a conformation of 2 with these protons in a plane.

For compound 3, the ^{1}H NMR spectrum showed signals of two CH_2 groups, one of which, with signals at δ 1.81 and 1.86, had couplings to H-5. In the ^{13}C NMR spectrum, signals for two quaternary carbons, assigned to C-2 and C-3 were observed. Their respective chemical shift, δ 95.31 and 96.25, indicated that both carbonyls were in the hydrate form, 3a.

Table 2

$^1H,^1H$ Coupling constants ($|J|$/Hz) for AF (1), reaction intermediates (2-4), 1',4-anhydrosaccharinic acid (5), and derivatives

Cpd	1	2	3a	4	5	5a	5b	6	6a	6b
Solv.	D_2O	D_2O	D_2O	D_2O	D_2O	$CDCl_3$	$CDCl_3$	D_2O	$CDCl_3$	$CDCl_3$
1a,1b	11.9	17.0								
1b,5		2.1								
1'a,1'b					9.4	10.7	9.8	10.0	10.1	10.5
3a,3b					13.3	13.4	12.5			
3(a),4					9.9	9.8	10.8	8.0	6.7	7.8
3b,4					6.1	6.1	6.1			
4a,4b	-		13.4	14.9						
4(a),5	8.8	2.1	10.5	<1						
4,5a					6.3	6.3	4.9	5.5	6.8	4.5
4,5b					3.4	3.3	3.9	3.3	3.4	3.0
4b,5			3.6	14						
5a,5b					12.2	11.9	10.7	11.9	11.9	10.8
5,6a	2.1	3.3	2.4	3.0						
5,6b	6.6	5.8	7.1	5.8						
6a,6b	12.4	12.0	12.0	12.8						

When AF was treated with 1 M NaOD for 10 min, no signals for olefinic protons were observed in the ^1H NMR spectrum. Instead, this showed a rather simple spin-system, indicating that only one compound, **5**, had formed. In order to identify compound **5**, the experiment was repeated on a larger scale, using 1 M NaOH in H_2O. After 10 min, the solution was neutralized with an ion exchange resin and then concentrated to a syrup, which was analysed. A FAB-mass spectrum showed that **5** had a molecular mass of 162 according to the negative ion at *m/z* 161 corresponding to $[M-H]^-$. From the ^1H and ^{13}C NMR spectra (Tables 1 and 2), which were analysed with the help of 2D NMR and DEPT experiments, it was possible to establish the structure. In the ^{13}C NMR spectrum, signals for a quaternary, a carboxyl, a methine, and three methylene carbons were observed. The ^1H NMR spectrum showed two spin-systems, one from an isolated methylene group and a second containing all other protons connected through scalar couplings. These data identified **5** as 1',4-anhydro-3-deoxy-2-*C*- (hydroxymethyl)-D-pentonic acid [3-hydroxy-5-(hydroxymethyl)oxolane-3-carboxylic acid], a compound that has been isolated as a product from the alkaline degradation of reducing sugars [7,14].

When **5** is generated from **3** by a benzilic acid rearrangement, C-2 could adopt either the (*R*)- or the (*S*)-configuration according to which side of the carbonyl group the migrating bond is attacking. In order to determine the stereochemistry of C-2, the acetylated (**5a**) and the methylated (**5b**) derivatives were synthesized, as these have protons close in space either above or below the plane of the ring and thus should give rise to NOE. In the ^1H NMR spectra of **5a** and **5b**, the signals were separated well enough to permit NOE difference experiments. In a series of experiments, the resonance frequencies of selected protons were irradiated. Presaturation of H-3b of **5b** at δ 2.26 caused increased intensities of the signals from H-3a, H-4, and the *O*-CH_3 group at δ 3.31, showing proximity of these protons to H-3a. When the *O*-CH_3 group at δ 3.31 was presaturated, the H-1'a and H-3b signals were increased, and when H-1'a was presaturated the signals from H-1'b and the *O*-CH_3 group at δ 3.31 were increased. When H-4 was presaturated, increased intensities were observed for the signals from H-3b, H-5a, and H-5b. These data are in accordance with H-1'a, H-3b, H-4, and the *O*-CH_3 group, giving a signal at δ 3.31, being below the plane of the ring. This shows that compound **5** is 1',4-anhydro-3-deoxy-2-*C*-(hydroxymethyl)-D-*threo*-pentonic acid [(3*R*,5*S*)-3-hydroxy-5-(hydroxymethyl)oxolane-3-carboxylic acid].

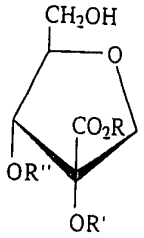
For the acetylated derivative **5a**, no contacts giving rise to increased intensities of the acetyl protons could be detected. This could be due to the longer distance between these protons and the ring protons.

To obtain more information on the reaction mechanism, alkaline transformation of ^{13}C-substituted AF (1-^{13}C and 2-^{13}C) to **5**, was carried out under the same conditions as mentioned above. When (1-^{13}C)-AF was used, a ^{13}C NMR spectrum showed that the product was ^{13}C-substituted at C-1', i.e., the methylene carbon adjacent to the ring oxygen. Using (2-^{13}C)-AF as starting material, the ^{13}C NMR spectrum of the product showed the strong signal at δ 179.91 indicating that the carboxyl carbon (C-1) was ^{13}C-substituted. This result shows that the carboxyl carbon in compound **5** originates from C-2 of the original AF and consequently, the hydroxide ion attacks at C-2 of the dicarbonyl compound **3**, forcing the C(1)-C(2) bond to migrate in the rearrangement (Scheme 1).

When D_2O was used as solvent, the ^1H NMR signals from the deoxy protons at δ 2.14 and 2.04 showed a different coupling pattern, lacking the geminal coupling. The signals had also lower intensities, only 27 and 40 %, showing that in addition to the incorporation of one deuterium atom of the position-4 when the enol form **2** rearranges to the keto form **3**, there is some further keto-enol tautomerization resulting in more deuterium substitution.

Reducing sugars have been determined by the spectrophotometric assay of the coloured product formed in the reaction of 3,5-dinitrosalicylic acid (DNS) with a reducing sugar in alkali, a method developed by Sumner [15]. This technique has been used for the detection and quantification of AF [16]. The reaction of AF with the DNS reagent was complete in less than 10 min at room temperature. This is in sharp contrast to the reaction with reducing sugars, such as glucose and maltose, for which the reaction with DNS reagent needs 100°C to be complete in that time [17].

When AF was treated with aqueous 1 M NaOH in the presence of DNS, one main product, **6**, was isolated. A ^{13}C NMR spectrum of **6** showed six lines (Tables 1 and 2), as for compound **5**, but the chemical shift for the C-3 signal (δ 84.13) indicated that this carbon is hydroxylated in compound **6**. According to the chemical



6

6 R, R',R''= H **6a** R= H; R', R''= Ac **6b** R, R', R''= Me

shifts and coupling constants of the spin-systems observed in the ^{1}H NMR spectrum, assigned by various 2-D experiments, the product was a 1',4-anhydro-2-*C*-(hydroxymethyl)-D-pentonic acid. The size of the coupling constant (8.0 Hz) between H-3 and H-4 indicated a *trans* relationship of these protons. In order to confirm the stereochemistry of compound **6**, the acetylated (**6a**) and the methylated (**6b**) derivatives were synthesized and subjected to NOE difference experiments. In a series of experiments, the resonance frequencies of selected protons were irradiated. For both derivatives, presaturation of H-3 caused increased intensities of the signals from H-5a and H-5b, whereas no effect on the H-4 signal could be observed. This result confirms that H-3 and H-4 are *trans*-related. When the *O*-CH_3 group at δ 3.48 in **6b** was presaturated, the H-1'a signal was increased, and when H-1'b was presaturated, the ester CH_3 group at δ 3.88 was increased in addition to the H-1'a signal. The same proximities were shown by presaturation of H-1'a and the ester CH_3 group, with increased intensities of the signals from the *O*-CH_3 group and H-1'b, respectively. When H-4 was presaturated, increased intensities for the signals from the 5-protons were observed. These results show that compound **6** is 1',4-anhydro-2-*C*-(hydroxymethyl)-D-arabonic acid.

The mechanism for the formation of compound **6** from the reaction of AF with DNS in aqueous NaOH could be the same as that for the formation of the acid **5** from the alkaline degradation of AF. The 3-OH group must be generated from **2**, as compound **6** could not be obtained when using component **3** or **4** as starting material. The 1'-methoxy derivative of **6** was isolated from the reaction of methyl β-D-glucopyranoside [18] in alkali in the presence of oxygen and hydrogen peroxide.

Acknowledgements

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